Differential effects of fatty acids on the endothelium

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Differential effect of fatty acids on the endothelium

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For Dad, Mum,
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Pour toute la famille

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Abstract

Background: Endothelial dysfunction is a major factor in the development of atherosclerosis, thrombosis and heart disease. Evidence suggests dietary fat composition may modify cardiovascular risk, as well as surrogate markers of cardiovascular risk such as blood pressure, arterial stiffness and endothelium-dependent vasodilation. **Aim**: To investigate the impact of dietary fat composition on endothelial function and associated markers of vascular health. **Methods**: The effects of oils rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were separately investigated in a parallel-design, placebo-controlled randomised controlled trial (n=48, 6 weeks, 2.9 g/d), carried out in free-living healthy young men. Following a 2 week run-in period taking placebo capsules (olive oil), participants underwent baseline measurements of finger capillary density, endothelial progenitor cell numbers (EPC), platelet-monocyte aggregate numbers (PMA), ambulatory blood pressure (ABP), pulse wave analysis (PWA), digital volume pulse analysis (DVP), and gave blood samples for plasma lipid, glucose, insulin, nitric oxide metabolites (NOx) and isoprostanes. The same measurements were made at the study endpoint, 6 weeks. An *in vitro* investigation of the effects of physiologically-relevant fatty acid profiles on microvascular endothelial cell nitric oxide and prostacyclin production was also performed. **Results**: Neither EPA nor DHA supplementation influenced EPCs, capillary density, PMA, ABP, PWA, DVP or plasma cholesterol, triacylglycerol, glucose, insulin, NOx or isoprostanes compared to placebo. However, ambulatory night-time heart rate was increased following EPA supplementation compared to DHA. Furthermore, both EPA and DHA decreased plasma non-esterified fatty acids (NEFA) compared to placebo. The *in vitro* investigations suggested that the composition of circulating NEFA may differentially affect endothelial function in the microvasculature. **Conclusion**: Dietary EPA and DHA at relatively high doses do not improve a number of novel markers of vascular function, including microvascular function and a marker of endothelial repair in young healthy men. EPA and DHA have differing effects on heart rate during sleep, suggesting that further research is required into the possible adverse effects of higher doses of individual marine fatty acids in at-risk individuals. Further work is required to elucidate the role of physiological fatty acid profiles on endothelial function.

Author's contribution

The author was responsible for cell culture, ran western blot analysis, prostacyclin measurements and nitrate/nitrite assays.

The author and Dr Aseel Alsaleh (PhD student at the time) co investigated the EPA and DHA trial, which involved recruiting and screening subjects, capillaroscopy, flow cytometry analysis, and blood sample analysis.

The author statistically analysed and interpreted the data from all the studies and composed the present thesis, in discussion with Dr Wendy Hall, Prof Toms Sanders and Peter Milligan. I am very grateful to all of them for their help and support.

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Abbreviations

6-keto prostaglandin $F_{1\alpha}$	6-keto $PGF_{1\alpha}$
Acetylcholine	Ach
Acyl coenzyme A oxidase	ACOD.
Acyl coenzyme A synthetase	ACS
Acyl-CoA oxidase	ACOD
Adenosine triphosphate	ATP
Adenylate cyclise	AC
Advanced glycation products	AGE
Albumin	ALB
Alkaline phosphatase	ALP
Allophycocyanin	APC
Ambulatory blood pressure	ABP
Aminomethylpropanol	AMP
Ammonium persulfate	APS
Analysis of covariance	ANCOVA
Angiotensin converting enzyme	ACE
Angiotensin receptor	AT-1
Anhydrous potassium hydroxide pellets	KOH
Apoproteins	Apo
Arachidonic acid	AA
Aspartate transaminase	AST
Asymmetric Dimethylarginine	ADMA
Augmentation index	AIx
Augmentation pressure	ΔP
Bicinchoninic acid	BCA
Blood pressure	BP
Brachial–ankle	ba
Pentafluorobenzyl bromide	PFBBr
Bromocresol green	BCG
Bromophenol blue	BPB
Butylated hydroxytoluene	BHT

Calcium chloride	$CaCl_2$
Cardiovascular disease	CVD
Chelate copper	Cu
Cholesterol esters	CE
Circulating progenitor cells	CPC
Clinical Pathology Accreditation Ltd.	CPA
Coronary heart disease	CHD
Cyclic adenosine monophosphate	cAMP
Cyclic guanosine monophosphate	cGMP
Cyclooxygenase	COX
Cytochrome P450 enzymes	CYP450
Delipidised FCS	DFCS
Diastolic BP	DBP
Diastolic pulse contour analysis	DPCA
Digital converter	ADC
Digital volume pulse	DVP
Dimethyl sulfoxide .	DMSO
Dipotassium hydrogen ortho phosphate	K_2HPO_4
Docosahexaenoic acid	DHA
Docosapentaenoic acid	DPA
Eicosapentaenoic	EPA
Electrocardiogram	ECG
Endothelial basal medium	EBM
Endothelial Cell Growth Medium MV	ECGM-MV
Endothelial cell growth supplement	ECGS
Endothelial dysfunction	ED
Endothelial function	EF
Endothelial nitric oxide synthase	eNOS
Endothelial progenitor cells	EPC
Endothelin receptor	ET-R
Endothelin-1	ET-1
Endothelium-derived hyperpolarising factor	EDHF
Endotoxin	ETX

Enhanced chemilumescent	ECL
Enzyme immunoassay	EIA
Enzyme linked immunosorbent assay	ELISA
EPA and DHA trial	EDT
Epoxydocosapentaenoic acid	EDP
Epoxyeicosatetraenoic acid	EEQ
Ethylenediaminetetraacetic acid	EDTA
Fatty acids	FAs
Flow mediated dilation	FMD
Fluorescein isothiocyanate	FITC
Fluorescence	FL
Fluoride/oxalate	FX
Foetal calf serum	FCS
Food and Agriculture Organization (of the United Nations)	FAO
Forearme blood flow	FBF
Forward scatter	FS
Full Blood Count	FBC
G proteins	Gs
Gamma-glutamyl transferase	GGT
Gas chromatography	GC
Glucose-6-phosphate dehydrogenase	G6PD
Glyceryl trinitrate	GTN
Guanosine triphosphate	GTP
Guanylate cyclase	GC
Heart rate	HR
Hepes Buffer Saline	HBS
High density lipoproteins	HDL
Homeostatic model assessment	HOMA
Horseradish peroxidase	HRP
Human dermal microvascular cells	HDMEC
Hydrochloric acid	HCL
Hydrogen peroxide	H_2O_2
Hydroxydocosahexaenoic acid	HDoHE

Hydroxyeicosapentaenoic acid	HEPE
Immunoglobulin G- Horseradish peroxidase	IgG-HRP
Inducible nitric oxide synthase .	iNOS
Intercellular adhesion molecule	I-CAM
Interleukin-	IL
Intermediate-density lipoprotein	IDL
Intima media thickness	IMT
Intracellular adhesion molecules	ICAM
Iso-8-Prostaglandin $F_{2\alpha}$	iso-8-$PF_{2\alpha}$
Kinase insert domain receptor	KDR
L-arginine	L-Arg
Leukotrienes	LT
L-Glutamine with Penicillin/Streptomycin	L-Gln-Pen-Strep
Linoleic acid	LA
Lipoxygenase	LOX
Lithium heparin	LH
Low density lipoproteins	LDL
Macrophages	MΦ
Magnesium sulphate	$MgSO_4$
Magnetic resonance imaging	MRI
Mean arterial pressure	MAP
Metalloproteinase-9	MMP-9
Mitogen activating protein kinase / phosphatidylinositol 3-kinase / protein kinase B	MAPK/PI3K/PKB
Monoclonal Ab	mAb
Monounsaturated FAs	MUFAs
Mouse immunoglobulin	MOPC31
National Institute of Standards and Technology	NIST
Negative chemical ionization	NCI
NG-methyl-L-arginine	L-NMMA
Nitrate	NO_3^-
Nitric oxide	NO
Nitrite	NO_2^-
NN-Diisopropylethylamine	DIPEA

NNN'N'-tetramethylethylenediamine	TEMED
NO synthase	NOS
Nominal molecular weight limit	NMLW
Non-esterified fatty acids	NEFA
Nuclear factor	NF
Nucleated Red Blood Cell Count	NRBC
Para-nitrophenyl phosphate	PNPP
Peak-to-peak time	PPT
Peroxidase	POD
PGI synthase	PGIS
Phase contrast-magnetic resonance imaging	PC-MRI
Phosphatidylinositol 3-kinase	PI3K
Phospholipase C / adenylate cyclase.	PLC/AC
Phospholipase C / protein kinase C	PLC/PKC
Phospholipids	PL
Photomultiplier tubes	PMTs.
Phycoerythrin	PE
Physiological levels	PP
Platelet monocyte aggregates	PMA
Polyclonal antibody	pAb
Polyunsaturated fatty acids	PUFA
Polyvinylidene fluoride	PVDF
Potassium carbonate	K_2CO_3
Potassium chloride	KCl
Potassium diHydrogen ortho Phosphate	KH_2PO_4
Programmable Temperature Vaporization	PTV
Prostacyclin	PGI2
Prostacyclin receptor	IP
Prostacyclin synthase	PGIS
Prostaglandin $F_{1\alpha}$	$PGF_{1\alpha}$
Prostaglandin H_2	PGH_2
Protein kinase B	PKB
PUFA:SFA	P:S ratio

Pulse pressure	PP
Pulse wave velocity	PWV
Pyrophosphoric acid	PPi
Quantitative insulin sensitivity check index	QUICKI
R squared	R^2
Reactive oxygen species	ROS
Receptor	R
Red blood cell velocity	RBCV
Reflection index	RI
Saturated fatty acid	SFA
Selective ion monitoring	SIM
Side scatter	SS
Sodium Azide	NaN_3
Sodium chloride	NaCl
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS PAGE
sodium dodecyl sulphate	SDS
Sodium methoxide	$NaOCH_3$
Sodium nitroprusside	SNP
Standard deviations	SD
Stiffness index	SI
Systemic arterial compliance	SAC
Systolic BP	SBP
Systolic pulse contour analysis	SPCA
Thin layer chromatography	TLC
Thromboxane A_2	TXA_2
Thromboxanes	TX
Total cholesterol	TC
Total protein	tp
Thromboxane A_2 receptor	TP
Transforming growth factor β	TGF-β
Triglyceride	TAG
Tumor necrosis factor α	TNF_α
Vascular endothelial growth factor receptor-2	VEGFR-2

Vascular smooth muscle cell	VSMC
Velocity of red blood cells	RBCV
Very low density lipoproteins	VLDL
World Health Organization	WHO
α-linolenic acid	ALA

Chapter 1 General introduction

This thesis is composed of *in vivo* and *in vitro* work which explores the mechanisms involved in the modulation of cardiovascular disease (CVD) risk factors by dietary fatty acids (FA). CVD, the major cause of death worldwide, involves atherosclerosis, inflammation and thrombosis, in which the endothelium plays an important role. Endothelial dysfunction (ED) is indeed the earliest stage in the pathogenesis of atherosclerosis known so far. It is often encountered in insulin resistant states such as type 2 diabetes and obesity, and has been associated with high levels of non-esterified fatty acids (NEFA) in plasma. Morbidity and mortality of all causes, especially CVD, have multifactorial causes from genetic background to environmental factors. Among the latter, diet is one of the most important ones. The Seven Country study was the first of its kind, examining the relationship between lifestyle, diet, coronary heart disease (CHD) and stroke. From this study arose the importance of the relationship between dietary fats and the risk of heart disease (Keys, et al., 1966). Amongst the different types of fats consumed, n-3 polyunsaturated fatty acids (PUFA), especially those from fish oils, show clear health benefits and have become of particular importance.

This thesis set out the hypothesis that dietary fatty acid composition modulates endothelial and vascular function, as well as related biological processes involved in the development of CVD. The following section aims at defining the current knowledge and uncertainties regarding the role of dietary fats on endothelial and vascular function in humans.

1.1 Dietary fats and cardiovascular disease (CVD) risk

1.1.1 The different types of fatty acids (FA) and their dietary sources

Fatty acids (FAs) are aliphatic monocarboxylic acids that usually contain an even number of carbons (from 2 to 28) in their side chain. They can be classified into three categories depending on their degree of unsaturation (Harvey, et al., 2005, Ratnayake and Galli, 2009).

Saturated FAs (SFAs) do not contain any double bond and are found universally in all fats. They include short chain FAs (less than 8 carbons), medium chain FAs (from 8 to 12 carbons), long chain FAs (14-20 carbons) and very long chain FA (22 carbons and more). They are mainly provided by animal and especially ruminant dairy fats. Many vegetable oils contain as little as 10% saturated fatty acids but some tropical fats e.g. coconut oil, palm oil and cocoabutter are rich sources. The hydrogenation of vegetable oils also results in an increase in the proportion of SFA.

Monounsaturated FAs (MUFAs) contain one double bond in their side chain and are universally present in most fats, from both animal and plant sources. Oleic acid (C16:1 *n*-9) is the main MUFA and is found in particularly high proportions in olive oil and canola oil, as well as high oleic sunflower oil and safflower oil. Palmitoleic acid (C16:1 *n*−7), synthesized from palmitic acid, is present in a variety of animal, vegetable and marine oils, especially in liver and in fish oil.

Polyunsaturated FAs (PUFAs) contain two or more double bonds that are methylene interrupted and in the cis configuration. The two main families are the *n*-3 and the *n*-6 PUFAs.

n-6 PUFAs include linoleic acid (C18:2 n-6) that is found in many vegetable oils such as safflower, poppy seed, walnut, sunflower, olive and palm oils. It is referred to as an essential FA: it must be obtained from plant materials as animals and humans are not able to synthesize it. It is a source of arachidonic acid (AA, C20:4 n-6), which can also be provided in the diet by animal fats, liver, eggs and fish. γ-linolenic acid (C18:3 n-6) is an intermediary in the formation of arachidonic acid and is a very minor component of animal tissues.

n-3 PUFAs include α-linolenic acid (ALA, C18:3 n-3) that is mainly found in seed oils, notably those of rapeseed, soybeans, walnuts and flaxseed. It is an essential FA and is the precursor of eicosapentaenoic (EPA, C20:5 n-3), docosapentaenoic (DPA, C22:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) (Tapiero, et al., 2002). The conversion rate from ALA to longer *n*-3 PUFA is limited in humans (Burdge and Wootton, 2002, Gerster, 1998), but EPA and DHA can be provided by fish oils such as cod liver, herring, mackerel or salmon oils. Long-chain (LC) *n*-3 PUFA refer to ALA+EPA+DPA+DHA, while very long chain (VLC) *n*-3 PUFA refer to EPA+DPA+DHA.

EPA competes with AA through the cycloxygenase (COX) and lipoxygenase (LOX) pathways, leading to a set of lipid mediators that improve vasodilation and decrease inflammation as well as aggregation (Figure 1-1). Upon the action of aspirin, EPA and DHA can be converted by the COX and LOX into similar but different families of resolvins, E and D-series, respectively (Burdge and Calder, 2005). In addition, both EPA and DHA compete with AA for the cytochrome P450 enzymes, leading to the formation of important mediators of vasodilation (Konkel and Schunck).

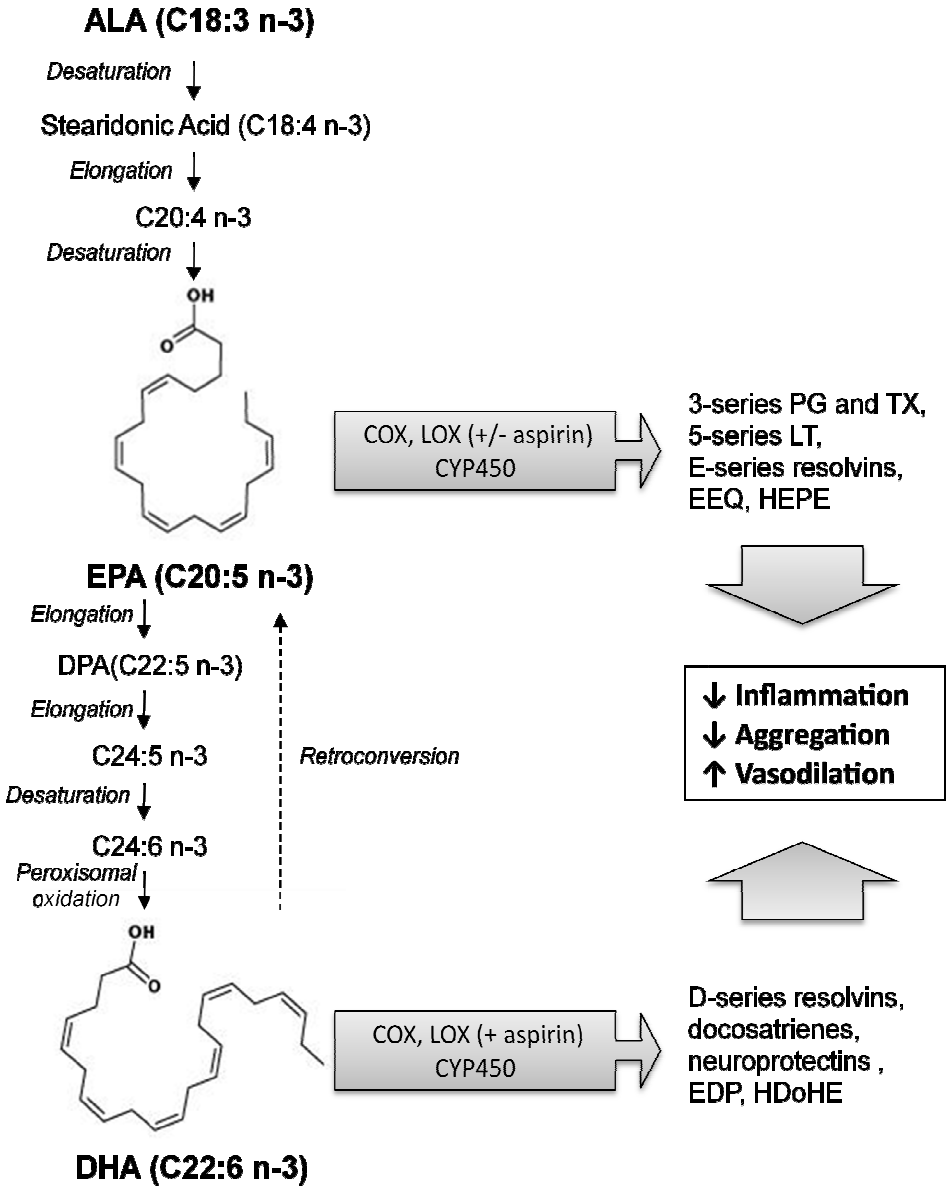

Figure 1-1: Formation of EPA and DHA and their metabolites – Outline (Balligand, et al., 1995)

ALA, α-linolenic acid ; EPA, eicosapentaenoic acid ; DPA, docosapentaenoic acid ; DHA, docosahexaenoic acid ; COX, cyclooxygenase ; LOX, lipoxygenase ; CYP450, cytochrome P450 enzymes ; PG, prostaglandins ; TX, thromboxanes; LT: leukotrienes ; EEQ, epoxyeicosatetraenoic acid ; HEPE, hydroxyeicosapentaenoic acid ; EDP, epoxydocosapentaenoic acid ; HDoHE, hydroxydocosahexaenoic acid.

1.1.2 Lipid metabolism and transport

Dietary fats are mainly composed of triglycerides (TAG) containing various long chain FA, as well as a small proportion of short- and medium-chain FA. The latter are volatile and water soluble; therefore they are directly taken up by the intestine capillaries and released into the portal vein. In contrast, long chain FAs are highly water insoluble and TAG must be emulsified and hydrolysed to monoacylglycerol and free FA prior to absorption. This process of digestion is divided into three steps: 1) emulsification in the stomach, 2) lipolysis and solubilisation with bile salts in the duodenum, 3) absorption into the enterocyte in the ileum (Bauer, et al., 2005).

The TAG, phospholipids (PL) and cholesterol esters (CE) - newly synthesized in the enterocyte – are transported into the blood stream by the lymph vessels. Since they are water insoluble, they must be bound to apoproteins (Apo) to form lipoprotein particles to circulate in the blood. Chylomicrons (large TAG rich lipoproteins) carry the esterified dietary derived FA from the enterocyte to peripheral tissues (muscle and adipose tissue) where TAG are hydrolysed through the action of lipoprotein lipase, attached to the capillary endothelium. The resulting non esterified FA (NEFA) and glycerol pass through the capillary wall where they can be used for energy or stored as TAG, while the remnants of chylomicrons are cleared by the liver. Unlike dietary fats, endogenous fats are combined with ApoB in the liver to form very low density lipoproteins (VLDL). Through the lipase hydrolysis, VLDL loses some of the TAG. They are progressively transformed into intermediate-density lipoprotein (IDL), and then low density lipoproteins (LDL), which are then taken up by the liver and peripheral tissues. By contrast high density lipoproteins (HDL) are delivered by the intestine and the liver as apoproteins (mostly ApoA-I and II) that are free or almost free of FA (Basso, et al., 2003, Brunham, et al., 2006). These nascent HDL particles subsequently recruit cholesterol and PL from the liver, as well as peripheral tissues. The mature HDL are returned to the liver, but can also transfer cholesterol to VLDL and chylomicrons (Rader and Daugherty, 2008, Ratnayake and Galli, 2009).

More than 90 percent of FAs found in the plasma are esterified (TAG, PL, CE) and contained in lipoprotein particles. NEFA released from TAG stored in adipose tissue, as well as some of NEFA released from circulating TAG and derived from *de novo* lipogenesis in the liver, are transported in the circulation bound to albumin, before being

cleared by the liver (Bergman, et al., 1971, Coppack, et al., 1992, Hellerstein, 1999). The type of fat consumed greatly influences the composition of these lipid fractions, which can be used as markers of fat intake. TAG mirror the dietary intake of the few hours or the last day and are subject to great variations (Durrington, et al., 1977, Moore, et al., 1977, Vessby, et al., 1980). CE are reflective of slightly longer term intake, i.e. ~3-5 days (Zock, et al., 1997, Zuijdgeest-van Leeuwen, et al., 1999). PL reflect the intake over similar periods to CE but appear to be more repeatable and is the fraction that is most commonly used, despite lower concentrations (Seppanen-Laakso, et al., 2001, Zuijdgeest-van Leeuwen, et al., 1999). Noteworthy, circulating cell membranes have been used to reflect dietary intake in nutritional interventions, including platelet, inflammatory cell and erythrocyte membranes. The latter have a lifespan of 120 days and their FA composition has been suggested to be a better marker of fat intake than plasma FA (Arab, 2003), especially for DHA status (Sun, et al., 2007). Because of its low turnover, adipose tissue is considered as the best option to represent very long-term FA intake over years (Dayton, et al., 1966, Hodson, et al., 2008), with an estimated half life of at least 6 months or a year (Beynen, et al., 1980, Katan, et al., 1997, Strawford, et al., 2004). Importantly, PUFA are almost exclusively exogenous and are thus better biomarkers of fat intake than those that can be synthesised endogenously, i.e. SFA and MUFA (Arab, 2003).

It should be noted that the efficiency of absorption and metabolism may vary depending on the TAG structure (Berry, 2009), as well as the type of FA considered. For example, the proportion of oleic acid in PL, but not platelet or erythrocyte PL reflected change in MUFA intake (Hodson, et al., 2001). There is also evidence that EPA and DHA from fish oils are incorporated at different rates into CE, erythrocytes and adipose tissue (Katan, et al., 1997).

Nonetheless, the composition of lipid fractions represents a better index of FA intake that the traditional food questionnaires and has been proven to be greatly useful in relating FA intake to CVD risk, in both intervention and epidemiological trials.

1.1.3 Dietary habits and CVD risk: the importance of fats

Numerous epidemiological studies have investigated the relation between lifestyle and heart health over the past 100 years. Amongst them the Framingham Heart Study, launched in 1948 in Massachusetts (Dawber, et al., 1951), played a pioneering role in the

identification of CVD risk factors such as high blood pressure, high cholesterol, obesity, diabetes, smoking and physical inactivity (Castelli, 1984, Kannel, 1987, Kannel, et al., 1961). The Seven Countries Study, launched 10 years later, enrolled populations of men ages 40-59, in eighteen areas of seven countries, with follow-up for deaths in the cohorts up to the present day. The Seven Country study furthered the understanding of the relationship between heart disease and lifestyle, particularly the composition of the diet and especially fat intake (Keys, et al., 1966).

1.1.3.1 The western diet:

It was estimated that 72% of the energy intake of our diet – as consumed in western countries - is composed of food that would have been scarce or unavailable in the pre-agricultural hominin diet. This has led big differences in glycemic load, fatty acid composition, macronutrient composition, micronutrient density, acid-base balance, sodium-potassium ratio, and fibre content in contemporary "Western" diets compared to the hunter-gatherer diet that was consumed for the majority of human evolution (Cordain, et al., 2005). Our "western" pattern is characterized by a high intake of processed food, red meat, high-fat dairy products, eggs, and refined grains. In terms of fats, it contains higher proportions of SFA and *trans* FA, and a higher *n*-6;*n*-3 PUFA compared to the non-western diet such as the Japanese diet. The Seven Country study was the first to show that high SFA and *trans* FA intake are associated with an increased platelet activity and a higher risk of atherosclerosis and CHD (Kromhout, et al., 1995, Lo, et al., 2001, Oomen, et al., 2001). A reduction in dietary SFA was later shown to improve CVD risk factors such as total and LDL cholesterol (Lawson, et al., 1991), and hypertension (Chisalita and Arnqvist, 2004). However, the relation of SFA with higher CVD risk has come under criticism (Hoenselaar, 2012). In several randomised controlled trials, SFA was replaced by PUFA, and the ratio of PUFA:SFA (P:S ratio), rather than SFA *per se*, may responsible for the beneficial effect on CVD risk (Skeaff and Miller, 2009). In support of that, other studies have reported no effect of reduced SFA intake on CVD risk; and a recent meta-analysis of prospective epidemiologic studies including 347,747 subjects has shown that SFA intake had no effect on CHD risk (Siri-Tarino, et al., 2010). Accordingly, the replacement of 5% of total energy from SFA by PUFA has been estimated to reduce CHD risk by 42% and substituting SFA in the diet by PUFA, rather than carbohydrates, may be more beneficial with respect to CVD risk.

1.1.3.2 Mediterranean diet

A Mediterranean diet as consumed in Italy, Spain and Greece, is a collection of several dietary habits and emphasizes a high intake of vegetables, legumes, fruits, nuts, whole grains, and olive oil, as well as a moderate intake of fish and red wine. In contrast, the consumption of dairy products and red meat is relatively low. Epidemiological studies first investigated the influence of the whole diet, rather than a single nutrient, on CVD risk. The beneficial aspects of the Mediterranean diet first emerged in the late 60's from the Seven country study, which reported that deaths from CHD in the USA and Northern Europe greatly exceeded those in Southern Europe, even when controlled for age, BP, cholesterol, smoking, physical activity and weight (Keys, 1975, Keys, et al., 1966). This beneficial effect was soon attributed to the high consumption of MUFA (predominantly from olive oil) relative to SFA (less animal fats) (Keys, et al., 1986). From this time, epidemiological and intervention studies have flourished showing a beneficial effect of the Mediterranean diet, and particularly olive oil, on cardiovascular (CV) health, including lipid profiles, blood pressure, endothelial function (EF), glycemic control and insulin sensitivity (Alvarez Leon, et al., 2006, Esposito, et al., 2004, Gillingham, et al., 2011, Schwingshackl, et al., 2011, Schwingshackl, et al., 2011, Yu, et al., 1995). However, other components of the Mediterranean diet, including microconstituents such as phenol compounds, vitamin E and carotene, and n-3 PUFA from fish and nuts may also account for the cardioprotective effect of the Mediterranean diet.

1.1.3.3 The Inuit diet

While the Mediterranean diet is characterised by a complex pattern of dietary habits, the traditional Inuit diet consists mainly of marine animals and fish, and is therefore low in carbohydrates and rich in proteins and fats, particularly in n-3 PUFA. In the late 70's Dyerberg and Bang were the first to highlight the cardioprotective effect of dietary long chain *n*-3 PUFA present in oily fish in the Inuit population (Dyerberg, et al., 1975). The authors observed a low incidence of CVD in the Inuit population living in Greenland compared to the residual Danish population. This favourable effect was soon associated to the ability of *n*-3 PUFA to compete with *n*-6 PUFA, thus showing anti-thrombotic and hypotriglyceridemic properties (Dyerberg and Bang, 1978, Dyerberg and Bang, 1979, Goodnight, et al., 1982). Over the past 30 years numerous studies – epidemiological and

intervention trials - have confirmed the protective effect of fish oils on CVD (Marik and Varon, 2009, von Schacky and Harris, 2007) such as fatal CHD (He, et al., 2004, Hu and Willett, 2002), atherosclerosis (von Schacky, 2000) and stroke (He, et al., 2004, Keli, et al., 1994). The mechanisms by which fish oils improve CV health have been extensively investigated, showing anti-inflammatory, anti-arrhythmic, anti-aggregatory and possible insulin sensitising effects, as well as an improvement of vascular and EF (Calder and Yaqoob, 2009, Harris, et al., 2008, Johansson, et al., 2008).

However, since the 1970's when the cardioprotective effect of the Inuit diet was suggested, this population has decreased the consumption of local food, and imported products have increasingly featured in their diet, evolving towards a more 'westernised' diet, higher in SFA and refined carbohydrates (Deutch, et al., 2007, Sharma, 2010). This, in addition to a decrease in physical activity, has been postulated to explain a possible rise in CVD risk in this population, including hypertension and obesity (Chateau-Degat, et al., 2010).

1.1.3.4 Fish oils and cardiovascular health

- *Fish oils and CV events*

Despite fears that there may be substantial health risks to consuming oily fish such as salmon, comprehensive reviews and meta-analyses have clearly indicated that the beneficial effects of fish oils largely compensate for any risk due to the presence of heavy metals and organic pollutants (Mozaffarian and Rimm, 2006, Sioen, et al., 2007).

Meta-analyses have demonstrated that reduced tissue or blood levels of LC *n*-3 PUFAs, provide a better indicator of CVD risk than the n-6:n-3 ratio, especially levels of DHA, with an effect size of -0.77 on the occurrence of CHD events in prospective studies, ($P < 0.01$) (Harris, et al., 2007). The authors concluded that absolute amounts of n-3 PUFA, rather than n-6 PUFA or n-6:n-3 ratio were better predictors of CVD risk, a view supported by others (Hjelte and Nilsson, 2005, Horrobin, 2000, Wijendran and Hayes, 2004). Just as LDL is considered as a risk factor for coronary artery disease (CAD), the omega-3 index (erythrocyte EPA+DHA) has been proposed as a modifiable risk factor for sudden cardiac death (SCD), as it is determined by diet and probably also has a genetic component (Harris, et al., 2004, von Schacky and Harris, 2007).

Numerous meta-analyses have flourished in the past two decades investigating the effect of fish oils on the incidence of death from CVD and CV events, gathering both observational and intervention studies. Findings have demonstrated a significant dose-response relationship between risk for CHD death and intake (P = 0.03), with relative risk reductions of 37% at an average EPA + DHA intake of 566 mg/d (Harris, et al., 2008). This led to dietary recommendations which targeted an intake of 400 to 500 mg/d of EPA + DHA in the UK and other European countries (1.1.3.5, **Table 1-1**), achievable by diet alone with the consumption of one portion of oily fish per week. In patients with a history of myocardial infarction, a significant reduction in the rate of sudden cardiac deaths (-40%) was found only when patients were treated with at least 1g EPA+DHA/d, which was defined by the American Heart Association and the European Society of Cardiology as a target intake for secondary prevention (Weber, et al., 2006)

In a more recent review (See Figure 1-2), Mozaffarian and Wu have confirmed that consumption of fish or fish oil significantly reduces CHD mortality, including fatal myocardial infarction and sudden cardiac death, in populations with and without established CVD (Mozaffarian and Wu, 2011). Relationships with total CHD or nonfatal coronary syndromes were more modest, and the authors suggested that, at usual dietary doses, n-3 PUFA might principally reduce ischemia-related cardiac death (Siscovick, et al., 2003).

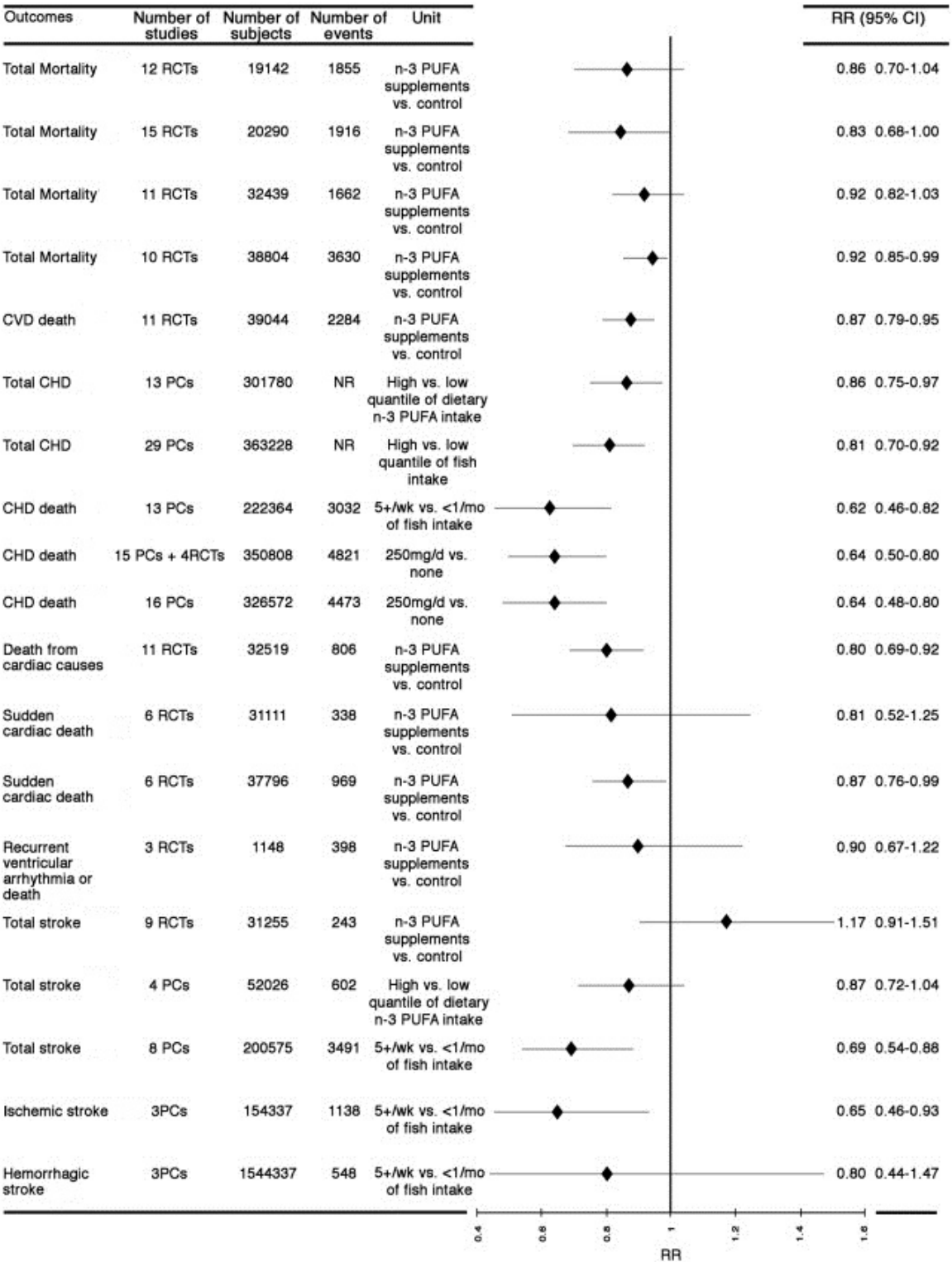

Figure 1-2: meta-analyses of studies of fish or long-chain n-3 PUFA consumption and risk of CVD outcomes, taken from (Mozaffarian and Wu, 2011)

prospective cohorts, PCs; randomised controlled trials, RCTs

♦ *Fish oils and CVD risk factors*

In randomised controlled trials, most effects of fish oils on CVD risk factors are only obvious at intakes usually in excess of 3g/d (See Figure 1-3) and not lower intakes which represent the amount consumed in human diets (Mozaffarian and Rimm, 2006).

The TAG lowering effect of FO is linearly dose-dependent up to at least 7g/d, but with a high variability of response, including a more important effect in individuals with higher baseline levels (Mozaffarian and Wu, 2011). A recent meta-analysis (58 RCT) concluded that each g/d of EPA+DHA reduces TAG levels by −5.9 mg/dL (95% CI : −2.5 to −9.3 mg/dL) overall, and by 1.7mg/dL (95% CI : -3.1 to -0.2 mg/dL) among subjects with TAG below the median 83 mg/dL(Mozaffarian and Wu, 2011).

The effect of fish oils on cholesterol levels is far less evident. Meta-analysis of 47 EPA+DHA supplementation studies (average 3.25 g/d) in hyperlipidemic subjects showed a very slight yet significant increase in LDL (+ 0.06 mmol/L, 95% CI: 0.03 to 0.09) and HDL (+ 0.01 mmol/L, 95% CI: 0.00 to 0.02), without affecting total cholesterol levels (Eslick, et al., 2009). A more recent meta-analysis of 11 RCT (*n* 485) concluded that algal DHA (median 1.68 g/d) increased LDL by 0.23 mmol/L (95% CI: 0.16-0.30) and HDL by 0.07 mmol/L (95% CI: 0.05-0.10) in healthy individuals (Bernstein, et al., 2012).

Beyond cholesterol levels, a few studies have reported a beneficial impact on lipoprotein size. At relatively high doses (3-4g/d), EPA and DHA were shown to increase HDL size (Chan, et al., 2006, Rambjor, et al., 1996, Wilkinson, et al., 2005), as well as LDL size (Griffin, et al., 2006, Mori, et al., 2000, Wilkinson, et al., 2005). sometimes without a significant change in lipoprotein levels (Agren, et al., 1996, Mori, et al., 2000). The effect of lower doses of fish oils on cholesterol metabolism remains uncertain, further research is needed and there is to date no meta-analysis on the effect on particle size, which could allow to quantify the dose effect. While the effect on cholesterol levels is unlikely to explain a reduction in CVD risk, even at high doses, their effect on lipoprotein particle size may be more relevant to CVD risk and remains to be fully elucidated and quantified.

Lowering blood pressure by 5 mmHg diastolic reduces the risk of stroke by an estimated 34% and ischaemic heart disease by 21% from any pre-treatment level, with no threshold (Law, et al., 2003). Recent meta-analyses indicate that EPA and DHA reduce both DBP and SBP, with a greater effect in hypertensive patients and those with high-

normal blood pressure, and the effective dose is likely to be at least 3g/d (Cabo, et al., 2012, Campbell, et al., 2012).

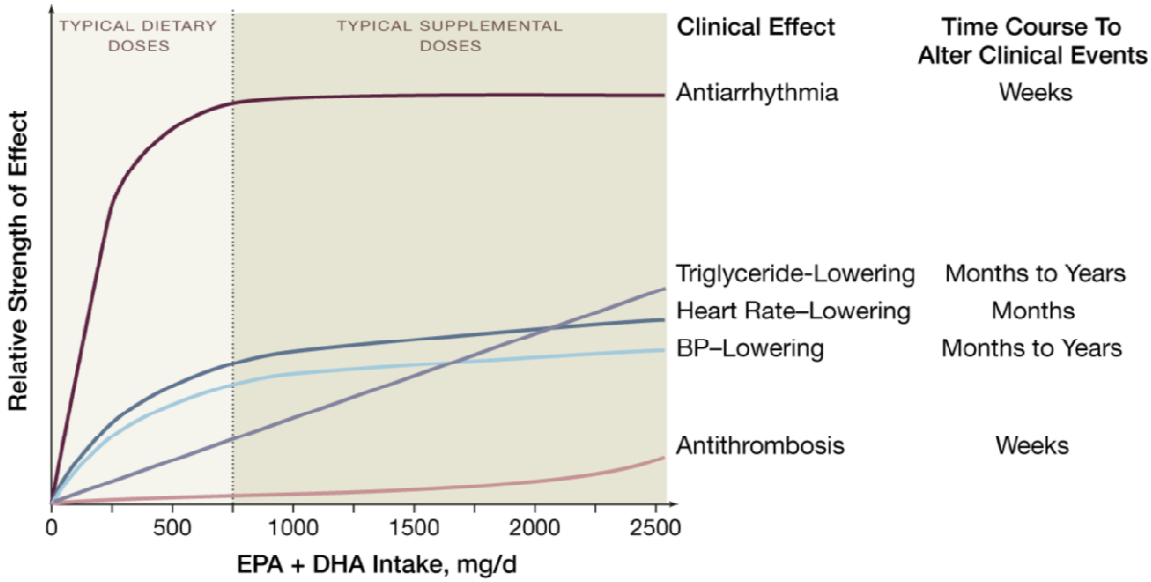

Figure 1-3: Relative strength of effect estimated from effects of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) on each risk factor and on the corresponding impact on cardiovascular risk, taken from (Mozaffarian and Rimm, 2006).

1.1.3.5 Dietary fats: Current recommendations

Low-fat/high-carbohydrate diets are widely promoted for weight management. However, reductions in total fat may include important reductions in essential FA, and the associated increase in carbohydrates - if dominated by simple sugars - can attenuate improvements in health outcomes resulting from weight loss (Woodley and Barclay, 1994). Rather than advising a low fat intake, current recommendations are for total fat to be <35% of total food energy intake (Department of Health, 1991), comprised of <11% SFA, <2% *trans* fatty acids, 6% PUFA, with the remainder of dietary fatty acids being MUFA (13% if other advice followed). Because the health benefits of ALA remain uncertain (Geleijnse, et al., 2010) and because humans can only convert ALA to EPA and DHA at a very low rate (Doughman, et al., 2007), it is currently recommended to consume EPA and DHA from marine sources. Current guidelines in the UK are to consume 2 portions of fish per week, one of which should be oily fish. This would increase the consumption of EPA/DHA to

0.45 g/day for (SACN, 2004),. Because of the toxin content of oily fish, such as mercury, upper limits were set by the Food Standard Agency, according to the National Institute for Health and Clinical Excellence (NICE) guidelines (NICE, 2008). Men, boys and post-menopausal women can eat up to four portions a week. Because of the risk of baby malformation, girls and women of child-bearing age, as well as pregnant women should eat one to two portions of oily fish a week. Table 1-1 summarises dietary fat recommendations – including EPA+DHA – in the USA, European countries and organisations.

Table 1-1 Overview of dietary recommendations for the intakes of total fat and fatty acids intakes for adults as set by different organisations, adapted from (EFSA, 2010).

Countries / Organisation	Total fat	SFA	TFA	*cis*-MUFA	PUFA	Cholesterol	EPA + DHA (mg/d)	References
United Kingdom	33 E%	<10 E%	< 2 E%	12 E%	6 E% with at least 1 E% LA and 0.2 E% ALA		450	(SACN, 2004)
Germany, Austria, Switzerland	30 E%	<10 E%	< 1 E%		Total PUFA: 7-10 E%, n-6 PUFA 2.5 E%, n-3 PUFA: 0.5 E%	< 300 mg per day	250	(DACH, 2008)
France	30-35 E%	<8 E%	< 2 E%	20 E%	4 E% of which 4 % LA, 0.8 E% ALA, 0.20 E% LCPUFA		500	(AFSSA, 2010)
The Netherlands	20-40 E%	< 10 E%	alap		<12 E% of which at least 2 E% LA, 1.0 E% ALA		450	(GR, 2006)
USA	20-35 E%	< 10 E%	alap		5-10 E% LA, 0.6-1.2 E% ALA		~160	(IoM, 2005)
WHO / FAO	15-30 E%	< 10 E%	< 1 E%		6-10 E%, of which 5-8 E% n-6 PUFA and 1-2 E% n-3 PUFA	< 300 mg per day	400-1000 (1-2 portion / wk)	(WHO/FAO, 2003)
Nordic Countries	25-35 E%	<10 E% (incl. TFA)		10-15 E%	5-10%, of which 1 E% n-3 fatty acids		450	(NNR, 2004)

SFA: Saturated fatty acids; TFA: *Trans* fatty acids; c*is*-MUFA: c*is*-monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; LA: Linoleic acid; ALA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; alap = as low as possible; E%: percent of energy; incl., including.

1.2 Vascular physiology and endothelial function

1.2.1 The arterial tree and the vessel walls

Blood flows away from the heart via arteries, which branch and narrow into arterioles, and continue to reduce size to become capillaries as they reach muscle or organs. Here the capillaries form a network throughout the tissue called capillary bed. Unlike vein and arteries, capillaries function is not to transport blood but allow the movement of substances, including gases (O_2, CO_2) and nutrients (glucose, amino acids) in and out of the capillary. After the tissue has been perfused, capillaries (containing blood enriched in CO_2, poorer in O_2 and nutrients) join and widen to become venules and then widen more to become veins, which return blood to the heart.

Although blood vessels differ in size, distribution and function, they share many common features in terms of structure. All vessels are made of three layers (Figure 1-4), going from the lumen to the most external layer (Krause, 2005):

- The *tunica intima* is the thinnest layer of the vessel. It is composed of a single layer of endothelial cells and a subendothelial connective tissue (collagenous and elastic fibres).

- The *tunica media*, the thickest layer, is made of connective tissue and vascular smooth muscle cells (VSMC) which are particularly abundant in arteries and control the calibre of the vessel. The tunica media also contains two layers of elastic fibres, the internal and external elastic *lamina*, which support the *tunica intima* and *adventitia*, respectively.

- The *tunica adventitia* is the outer layer and is made of connective tissue (mainly collagen), which contribute to the regulation of the calibre of the vessel.

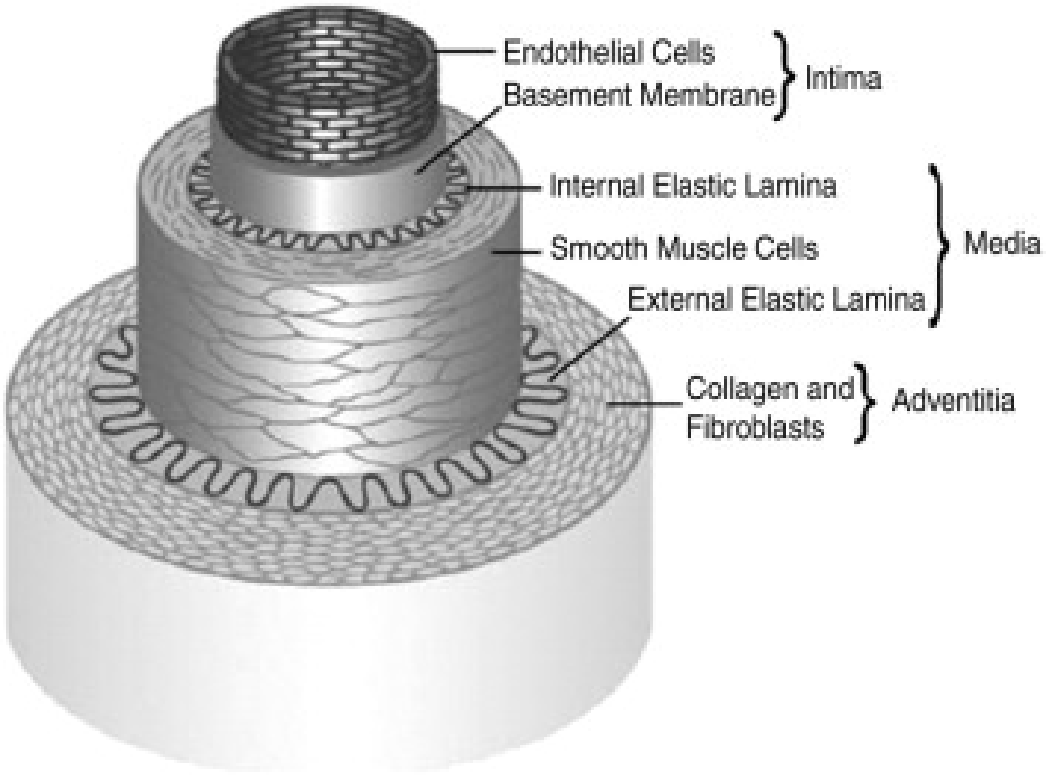

Figure 1-4 Drawing of arterial wall depicting the composition of three layers: intima, media, and adventitia (taken from (Sarkar, et al., 2007)).

The aorta and other large arteries (conduit and conducting arteries) serve as a blood reservoir able to stretch and recoil with the pumping action of the heart; their wall is thicker and largely made of elastic fibres. In contrast, the small arteries and arterioles (< 300 μm), which are involved in the regulation of blood flow and represent the major site of peripheral resistance, contain less elastic fibres and more VSMC as their diameter diminishes (Krause, 2005). Along the arterioles, the diameter gets smaller and the vascular wall thinner again. In the capillaries, the vessel wall is free of VSMC and only made of one layer of endothelial cells supported by a basement membrane, which allow the exchange of nutrients between the blood and tissue (Feihl, et al., 2006). Capillaries (4-12 μm) are just large enough to permit red blood cells to pass in single file. Their endothelium may be continuous (muscle, brain: gas exchange), fenestrated (digestive tract: passage of small molecules such as amino acid and sugar) or discontinuous (bone marrow, liver, spleen: holes are large enough to allow whole cells to pass). Veins possess the same three layers as the arteries but contain less VSMC and connective tissue, making their wall thinner than the one of arteries (Wagner and Frenette, 2008).

1.2.1 Vascular function and blood pressure (BP)

The macrovasculature is subject to high variations of pressure due to the pulsatile function of the heart while the microvasculature deals with a steady pressure and flow. During the contraction of the left ventricle (systole), blood pressure (BP) is mainly determined by the arterial stiffness of large arteries. When arteries get more rigid and less compliant, they lose the ability to adapt to the high pressure changes and this leads to a rise in systolic BP (SBP) within the vessel. In diastole (relaxation of the heart), the elastic recoil of the aortic wall propels the volume of blood to the periphery and BP diminishes. Diastolic BP (DBP) is determined by the intensity of the pressure wave reflected from the periphery, and increases when vascular resistance augments, i.e. when microvascular and ED occur. (Feihl, et al., 2009).

Hypertension is well-established as an independent CVD risk factor (Cacicedo, et al., 2004, Rizzo, et al., 2010, Staiger, et al., 2006, Symons, et al., 2009, Xiao-Yun, et al., 2009). Historically, high DBP was considered a better predictor than SBP, and allowed classifications for clinical treatment accordingly (Krogmann, et al., 2011). However in the past 15 years a paradigm shift has occurred towards SBP being a stronger predictor of CVD risk (Black, 1999). It arose from the Framingham study data that DBP (largely determined by vascular resistance) increases with age until age 50 to 60 when it starts falling (Franklin, et al., 1997). In contrast SBP, as well as the pulse pressure (PP = SBP – DBP), more influenced by arterial stiffness, appear to increase continuously with age. Thus the importance of treating elevated SBP is increasingly recognised in clinical practice and SBP appears to be a stronger predictor of CVD in elderly (Forstermann, et al., 1995, Franklin, et al., 1997). In fact PP, which reflects the stiffening of the aorta and large arteries, may be an even better predictor (Kaplan, 2000). Arterial stiffening provides a narrower and more rigid reservoir wherein there is a reduced compliance in the vessel walls to the forward travelling pressure wave during systole. In addition, the blood travels faster in stiff arteries and the reflected wave returns to the central aorta in mid systole rather than diastole. As a result, this augments the already high SBP while removing a major support of DBP, therefore increasing central PP. Thus treatment of isolated hypertension with anti-hypertensive drugs should be managed with care to avoid diastolic hypotension (Forstermann and Kleinert, 1995).

Whether arterial stiffness and/or PP are a better target than SBP to tackle CVD remains uncertain. ED, which is associated with arterial compliance and implicated in the dysregulation of vascular tone, is also a potential target. Techniques to assess vascular function have flourished in order to relate these outcomes to vascular risk and investigate their modulation upon drug or nutritional interventions.

1.2.2 Macrovascular function and arterial stiffness

The two main roles of conduit and conducting arteries are to transport the blood from the heart to all organs and buffering the pressure oscillations generated by the rhythmic left ventricular ejection. With ageing and the development of CVD, the vascular wall becomes thicker and stiffer. This phenomenon, called arterial stiffening, leads to an increase in blood velocity from the heart to the periphery and a reduction in arterial compliance, thus altering both the conducting and 'buffering' functions of large arteries, and augmenting SBP.

Arterial stiffening develops as a result of complex influences, both intrinsic (relative to the vessel wall structure and function) and 'extrinsic' (relative to haemodynamics and circulating factors such as salt, hormones or glucose). The complex mechanisms involved have been extensively reviewed (Zieman, et al., 2005) and entail structural changes, as well as the alteration of cell signalling and function. The inflammatory milieu is characterized by the infiltration of VSMC and the presence of macrophages and mononuclear cells, increased levels of metalloproteases (MMP), transforming growth factor (TGF)-β, intracellular adhesion molecules (ICAM) and cytokines (Lakatta, 2003), which alter the structure of both collagen and elastin. In addition, advanced glycation products (AGE's), which accumulate with ageing and diabetes, form irreversible cross-links between molecules such as collagen (Verzijl, et al., 2000). The overproduction of abnormal collagen, alteration of elastin and VSMC hypertrophy lead to an increase in intimal-medial thickness, thereby reducing the compliance of large conducting arteries (Johnson, et al., 2001, O'Leary, et al., 1999). In addition to structural changes, the alteration of smooth muscle tone also influences arterial stiffness. A number of locally derived factors such as nitric oxide, angiotensin II and endothelin-1 (produced by the endothelium), and reactive oxidant species (associated to ED), may alter VSMC function and contribute to arterial stiffening (Wilkinson and McEniery, 2004, Wykretowicz, et al., 2007). Furthermore other

circulating factors such as salt, glucose, insulin, neurohormones, natriuretic peptides and adiponectin also contribute to the functional regulation of arterial stiffness (Wilkinson and McEniery, 2004, Zieman, et al., 2005).

1.2.3 Microvascular function and vascular tone/peripheral resistance

In the intact vasculature, the largest pressure drop, and thus the highest resistance to flow, occurs between the arterioles and bigger arteries from which they originate. The microcirculation, which encompasses vessels < 150 µm in diameter, is therefore extremely important in determining peripheral resistance. In addition, by dissipating the pressure of arterial blood into smaller vessels, the arterioles and capillaries ensure a tightly controlled intra-capillary pressure, necessary for the normal exchange of substances between the capillaries and the peripheral tissues, as well as the preservation of the fragile capillary wall (Feihl, et al., 2009). The large number of capillaries makes up the largest volume of blood in the body and provides a large surface area for exchange substances, pressure in them is very low as blood is distributed out into a greater area, and high pressure may cause damage to the fragile capillary wall. They have no muscle in their walls and thus cannot adapt their diameter to pressure changes. Instead, the blood flow in the capillaries is regulated by the arterioles that serve them, containing VSMC. For decades it was thought that capillaries were static, uniform, semipermeable pipes, all made of a single layer of endothelium with no other function than maintaining a passive barrier between blood and tissue. In fact, capillaries are dynamic structures that participate actively in water, waste, gas and nutrient exchange, and are capable of sensing and responding to vasoactive stimuli to regulate in the blood flow to the tissue, and are able to adapt their number and arrangement in response the nutritional state of the tissue (Baldwin, 2000, Sarelius, et al., 2000). The regulation of VSMC contraction and vasodilation in the microvasculature, mainly mediated by the endothelium, may be transferred from the most distal capillaries towards the arterioles and subsequently to their feeding arteries (Bagher and Segal, 2011). Thus microvascular and EFs are crucial for the regulation of blood flow and peripheral resistance, and thus for the determination of DBP.

1.2.4 The vascular tone and the endothelium

1.2.4.1 Vasodilatation

The importance of the endothelium was first recognised in the 1980's by its effect on vascular tone, when Furchgott and Zawadzki demonstrated that acetylcholine-induced vasodilation required the presence of endothelial cells and a substance released by the endothelium (Furchgott and Zawadzki, 1980), which was later identified as nitric oxide (Moncada and Higgs, 2006). Nitric oxide, together with prostacyclin discovered earlier (Moncada, 2006) and a hyperpolarising factor yet to be defined (Bryan, et al., 2005), are the three main vasodilators produced by the endothelium (Figure 1-5).

Nitric oxide (NO) is synthesized from L-arginine via NO synthase (NOS), of which two types are present in the endothelium: the endothelial NOS (eNOS), constitutively expressed, and the inducible NOS (iNOS). eNOS is calcium-calmodulin dependent and produces NO under shear stress or physiological stimuli (e.g. insulin, acetylcholine or bradykinin) (Moncada and Higgs, 2006). In contrast, iNOS is calcium independent and generates large amounts of NO in response to inflammation (Moncada and Higgs, 2006). In physiological conditions, NO released by eNOS diffuses from endothelium to VSMC, where it activates the cytosolic form of guanylate cyclase, resulting in a rise of intra-cellular cGMP.

Prostacyclin (PGI_2) is synthesized via a COX, peroxidase and PGI synthase (PGIS) in the endothelial cell. It binds its receptor (IP) on the VSMC and induces its relaxation of through a cAMP dependent mechanism (Fetalvero, et al., 2007).

A third dilator factor has been named the endothelium-derived hyperpolarising factor (EDHF). Its identity and precursor remain controversial, but it is known to cause vasodilatation by hyperpolarising the VSMC, involving potassium channel activation, most often calcium-activated channels (Bryan, et al., 2005). Although the acronym suggests that EDHF is a unique molecule, it involves several different mechanisms. Arachidonic acid metabolites derived from the COX, LOX and cytochrome P450 pathways, H_2O_2, CO, H_2S and various peptides can be produced by the endothelium and contribute to the EDHF response (Feletou and Vanhoutte, 2009).

These three pathways lead to a decrease of intra-cellular calcium level, thus

phosphorylating the myosin light chains. Myosin is consequently inactivated, which decreases muscle contraction.

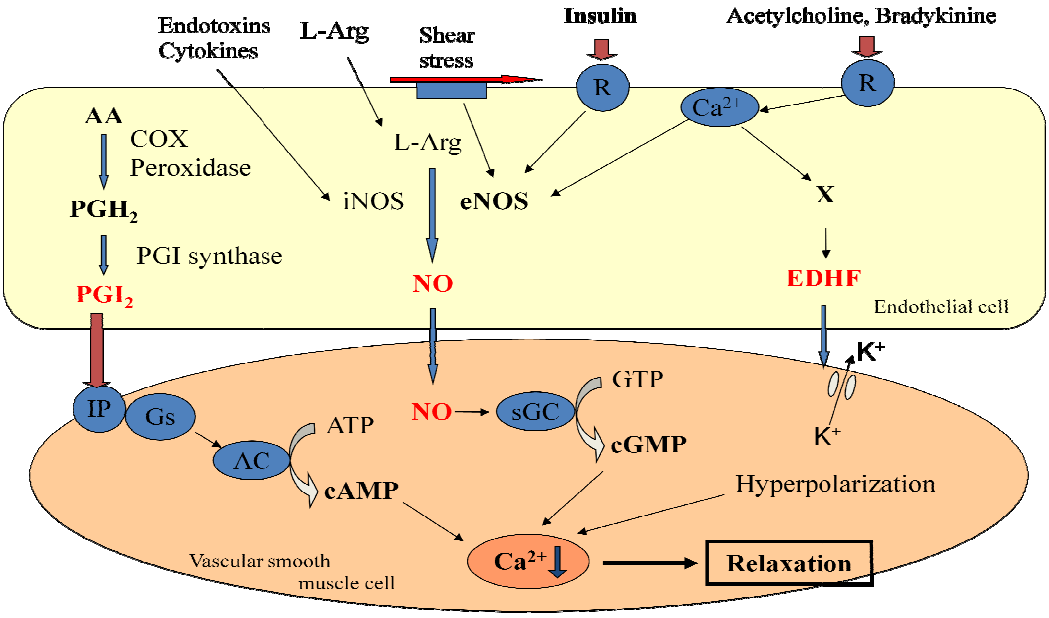

Figure 1-5 Outline of major mechanisms for vascular smooth muscle cell relaxation and contraction mediated by the endothelial cell.

Arachidonic acid (AA) is converted into prostaglandin H_2 (PGH_2) by cyclooxygenase (COX) and peroxidise, which leads to the formation of prostacyclin (PGI_2). Shear stress, as well as the binding of insulin and other vasoactive molecules (acetylcholine, bradykinine) to their receptors (R) increase intracellular Ca^{2+} levels. This results in the production of endothelium-derived hyperpolarising factor (EDHF) from an unknown substrate (X), as well as the stimulation of endothelial nitric oxide (NO) synthase (eNOS), producing NO from its substrate L-arginine (L-Arg). Inflammatory mediators (endotoxins, cytokines) may also stimulate inducible nitric oxide synthase (iNOS) to produce NO from L-Arg.,NO diffuses to the vascular smooth muscle cell where it activates soluble guanylate cyclase (sGC), causing an increase in cyclic guanosine monophosphate (cGMP) production and consequently a decrease in smooth muscle intracellular Ca^{2+} and relaxation. PGI_2 interacts with the PGI_2 receptor (IP), elevating cyclic adenosine monophosphate (cAMP) levels and decreasing intracellular Ca^{2+}, leading to relaxation of the smooth muscle. EDHF causes vasorelaxation by hyperpolarising vascular smooth muscle cells. ATP: adenosine triphosphate, GTP: guanosine triphosphate, AC: adenylate cyclise,

1.2.4.2 Vasoconstriction

In contrast, the endothelium is also able to induce vasoconstriction by secreting or converting several vasoconstrictive factors.

Thromboxane A_2 (TXA_2), which counters the effects of PGI_2, is synthesized from the same pathway (COX, peroxidase and TXA_2 synthase) and acts on VSMC through their G protein-coupled receptor TP (Norimichi, 2008). Although TXA_2 may be produced by the endothelium, its main production site is the platelet. More importantly, other prostaglandins generated by endothelial cells through COX-1 and/or COX-2, such as PGH_2, $PGF_{2\alpha}$, PGE_2, and paradoxically PGI_2, can all act through TP receptors and induce VSMC contraction (Félétou, et al., 2011).

Endothelin-1 (ET-1) is synthesized in the endothelium by the endothelin converting enzyme from a biologically inactive precursor called Big ET-1. It is one of the most potent vasoconstrictor peptide discovered so far. It is continuously released from the EC (constitutive pathway), inducing intense contraction of the underlying VSMC. It can also be released from EC-specific storage granules in response to external stimuli (Davenport and Maguire, 2006).

Angiotensin II is a hormone locally produced by angiotensin converting enzyme (ACE, expressed by the endothelium) from angiotensin I, converted from angiotensinogen by renin (Touyz and Schiffrin, 2000).

These molecules released from the endothelium then bind their specific receptors on VSMC and induce a subset of phosphorylation cascades that leads to a increase of intra-cellular calcium, thus stimulating the contraction of the cell.

1.2.5 Endothelial dysfunction

1.2.5.1 Definition of endothelial dysfunction

The endothelium was first seen as a passive barrier between blood and tissue, which when disrupted would lead to platelet aggregation to the subendothelial connective tissue and the proliferation of VSMC involved in the pathogenesis of atherosclerosis (Matsumoto, et al., 2010). The thin endothelial lining is now well known to act as a paracrine, autocrine and endocrine-like organ exerting many important biological functions, including the

regulation of vascular tone, cellular adhesion, haemostasis, smooth muscle cell proliferation and vessel wall inflammation. Beyond its anatomical disruption, the functional status of the endothelium emerged as crucial in the pathogenesis of CVD. Because the endothelium governs such a large array of functions, it is difficult to give a single definition to ED . Nonetheless, ED can be defined as an imbalance between the numerous molecules produced by the endothelium, i.e. an enhanced pro-inflammatory, pro-thrombotic, vasoconstrictive activities in the detriment of anti-inflammatory, anti-thrombotic and vasodilatory function (Bonetti, et al., 2003, Endemann and Schiffrin, 2004). The regulation of vascular tone being the main function of the endothelium, the impairment of vasodilation is the most commonly described alteration in ED.

A reduction of NO production by eNOS and NO availability is characteristic of ED, and is associated to an inflammatory and oxidant status of the endothelium. Reactive oxygen species (ROS) interact with NO to form peroxynitrites, which reduces NO availability (Koppenol, et al., 1992). Peroxynitrite contributes to the oxidation of LDL an important proatherogenic factor (Griendling and FitzGerald, 2003), and alter the reductase activity of eNOS (Milstien and Katusic, 1999), leading to more oxidative stress (Landmesser, et al., 2003). eNOS activity is also altered upon the action of pro-inflammatory cytokines, such as C-reactive protein, interleukin(IL)-1, IL-6 or tumor necrosis factor(TNF)-α (Kofler, et al., 2005, Venugopal, et al., 2002). In contrast inflammatory cytokines stimulate iNOS to produce high amounts of NO (Forstermann and Kleinert, 1995), which in the presence of ROS will lead to the formation of more peroxynitrites, thus increasing again oxidative stress (Xia, et al., 1996). Asymmetric Dimethylarginine (ADMA) and N^G-methyl-L-arginine (L-NMMA) are natural compounds released from cells upon protein degradation that accumulate in an inflammatory state and are associated with a higher incidence of CVD (Böger, et al., 2009, Kielstein and Zoccali, 2008). As analogues of arginine (eNOS substrate), ADMA and L-NMMA act as competitive inhibitors of eNOS and also contribute to the reduction of NO production (Cardounel, et al., 2007, Kielstein, et al., 2009). Vasoconstrictive factors, including angiotensin II, endothelin and COX-derived prostaglandins are also associated with ED and further the impairment of vasodilation (Mishra, et al., 2004, Versari, et al., 2009).

1.2.5.2 Insulin resistance and endothelial dysfunction

In diabetes additional mechanisms may contribute to ED due to the hyperinsulinaemia and lipotoxicity characteristic of insulin resistant states. In addition to its well known role in metabolism, insulin also exerts an important role in the vasculature. In physiological conditions, the main vascular action of insulin is to regulate vascular tone via the stimulation of the production of both NO and ET-1 (Muniyappa, et al., 2007). Insulin-induced NO production is due to the activation of the PI3K pathway, which also mediates insulin-induced GLUT-4 translocation in muscle cells and adipose tissue. Through the stimulation of NO production, insulin-mediated vasodilation is divided into two stages, affecting both the micro- and macrovasculature: 1) after a few minutes, terminal arterioles are dilated, which increases capillary recruitment, 2) then larger vessels are relaxed, which increases the blood flow to the tissue. Both of those NO-dependent mechanisms in skeletal muscle contribute to the increase of glucose disposal (Muniyappa, et al., 2007).

In insulin resistant state, the metabolic disorders are coupled with vascular and ED. Obesity is characterised by an increase in circulating NEFA levels that has been shown to alter the insulin-stimulated PI3K pathway, thus impairing glucose uptake. This leads to increased blood glucose concentrations and then to a compensatory hyperinsulinaemia that constitutes the early stage of type 2 diabetes development (Petersen and Shulman, 2006). As a consequence, hyperinsulinaemia overdrives the unaffected MAPK pathway, to the detriment of the PI3K pathway. In endothelium, this results in a reduction of NO production by eNOS associated with the increase of ET-1 production that are characteristic of ED (Figure 1-6) (Muniyappa and Quon, 2007).

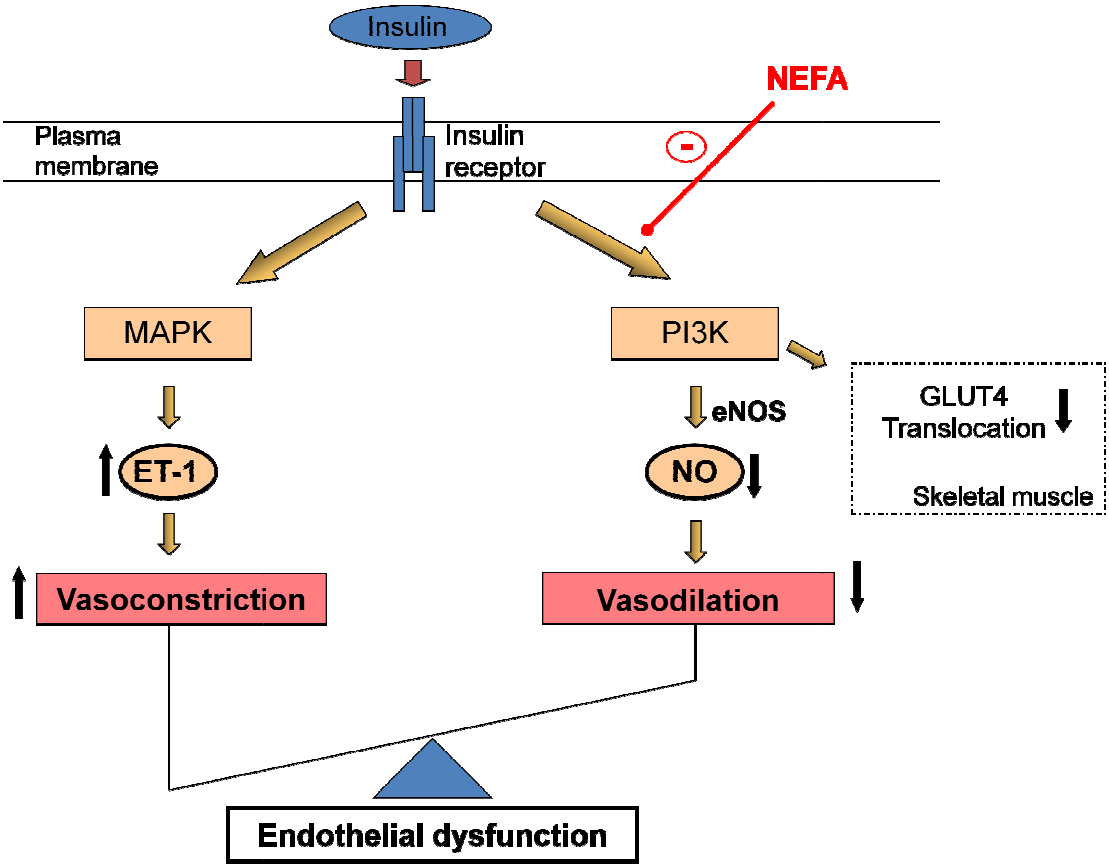

Figure 1-6 Pathway selective insulin resistance in phosphatidylinositol 3-kinase (PI3K) signaling in endothelial cell, adapted from (Muniyappa and Quon, 2007).
Elevated non esterified fatty acids (NEFA) inhibit PI3K, leading to a reduction of endothelial nitric oxide synthase (eNOS) activity and GLUT4 translocation. As a result nitric oxide (NO) and glucose uptake are reduced. In contrast, mitogen activated protein kinase; (MAPK) remains intact, and endothelin-1 (ET-1) increases compared to NO, leading to ED.

1.3 FA and vascular function

Several techniques, invasive and non invasive, as well as circulating markers may be used to assess vascular function, including arterial stiffness, EF and microvascular function. Here the main techniques are described, with an emphasis on non invasive techniques that are more commonly used in humans, and their potential modulation by dietary FA is discussed.

1.3.1 FA and macrovascular function

1.3.1.1 Assessment of arterial stiffness and compliance

Beyond the determination of pulse pressure, which represents a surrogate measure of arterial stiffness (Omura, et al., 2001), several techniques - both invasive and non invasive - have gathered great interest in the recent years to investigate the stiffness and compliance of the large arteries. Non invasive techniques fall into three categories: 1) measuring pulse wave velocity (PWV), 2) assessing the changes in diameter (or area) of an artery in response to distending pressure (=compliance, distensibility), and 3) analysing arterial pressure wave forms (Oliver and Webb, 2003).

- *Pulse wave velocity (PWV)*

PWV is the speed with which the pulse wave travels along a given length of artery. The arterial pulse wave is recorded at a proximal artery, such as the common carotid and at a distal artery, such as the femoral. The pulse wave travels between these two points along the aorta, an artery that is particularly prone to the development of arteriosclerosis and atherosclerosis. The distance and the time delay between these two sites allow the calculation of PWV in m/s. The time delay is obtained either by simultaneous measurement, or by independent measurements with electrocardiogram (ECG) - gating (Davies and Struthers, 2003).

Carotid–femoral PWV is the gold standard for assessing central arterial stiffness and a useful approach to diagnose atherosclerosis. Brachial–ankle (ba) PWV has also been developed as a surrogate measure of carotid–femoral PWV (Yambe, et al., 2004). Pressure-sensitive transducers (Asmar, et al., 1995), Doppler ultra-sound (Sutton-Tyrrell, et al., 2001) or applanation tonometry (Wilkinson, et al., 1998) may be used to detect the arterial pulse waves. Alternatively, PWV may be measured through the aorta using the phase contrast-magnetic resonance imaging (PC-MRI) technique (Metafratzi, et al., 2002).

- *Changes in vessel diameter (or area) to distending pressure*

Arterial compliance represents the relationship between changes in volume and changes in pressure during diastole (Stebbins, et al., 2008). Compliance and distensibility are defined as the absolute and relative changes in vessel diameter for a given change in distending pressure, respectively. The distending pressure is measured simultaneously and

is mainly determined by the mean arterial pressure (MAP) (McEniery, et al., 2007). Changes in diameters are most frequently measured by ultrasound in a number of arteries, such as the carotid, brachial, radial artery or aorta (Hoeks, et al., 1990, Meinders and Hoeks, 2004), as well as veins (Kölegård, et al., 2009). MRI may also assess the relative changes in cross-sectional area of a chosen segment, using ECG-triggered spin echo or gradient echo sequences (Metafratzi, et al., 2002).

- *Arterial pressure wave form analysis*

The most frequent analysis of pulse wave form consists in recording the pulse waveform recorded at a peripheral (usually radial) artery by applanation tonometry. Because changes in the contour of the pulse due to transmission along the upper limb are predictable and relatively constant from individual to individual, a generalised transfer function can be used to derive central aortic waveform form (Chen, et al., 1997). The systolic pulse contour analysis (SPCA) of the central waveform obtained allows the assessment of central BP, as well as the central arterial stiffness estimated by the augmentation index (AIx). AIx is the proportion of central pulse pressure that results from the arterial wave reflected from the periphery, and is thought to be largely determined by PWV, i.e. the stiffness of large arteries (Yasmin and Brown, 1999). However, accumulating evidence suggest that AIx is greatly influenced by other factors, such as the left ventricular ejection volume, the absolute systole duration or the vascular tone, determined by the diameter and elasticity of small muscular arteries and arterioles (Cecelja, et al., 2009, Protogerou and Safar, 2007, Wilkinson, et al., 2000).

Applanation tonometry can also be used to analyse the diastolic portion of the pulse wave. Diastolic pulse contour analysis (DPCA) uses a modified Windkessel model to assess the compliance of large and small arteries, usually referred to as C1 (proximal or capacitative compliance) and C2 (distal or oscillatory compliance) respectively (Nair, et al., 2005, Rietzschel, et al., 2001). Systemic arterial compliance (SAC) may be assessed by combining the use of Doppler to measure volumetric blood flow and applanation tonometry of the right common carotid artery. Similar to SPCA, an aortic pulse pressure is derived and the area under the curve in the diastolic portion is used to estimate compliance of the whole arterial tree (Stebbins, et al., 2008). SAC is defined as:

$$SAC = Ad/R(Pes-Pd)$$

where R is the total peripheral resistance (mean arterial BP / mean blood flow), Ad the area under the diastolic portion of the pressure waveform, Pes and Pd the the pressure at the end of the systolic and diastolic phase, respectively (Stebbins, et al., 2008).

- *Digital volume pulse*

Arterial pressure and volume wave forms may also be measured in the finger and closely mirror the radial wave form. Digital volume pulse (DVP) is recorded using plethysmography and can predict the arterial pressure pulse through a generalised transfer function (Millasseau, et al., 2000). Like the radial pressure pulse, the digital volume pulse is characterised by a forward (direct) wave and a backward (reflected) wave. The time delay between the two waves, and the amplitude of the reflected wave give information on the stiffness of large arteries (stiffness index, SI) and the vascular tone (reflection index, RI), respectively (Millasseau, et al., 2006).

- *Other techniques*

It should be noted that radial and aortic waveforms and pressures may also be measured invasively using fluid-filled catheters to give the indexes provided both SPCA (AIx) and DPCA (C_2) (Segers, et al., 2001, Söderström, et al., 2002). Pressure-diameter relationships in the aorta have also been determined invasively with simultaneous measurement of arterial pressure by using a luminal pressure transducer and dimensions by using intravascular ultrasound (Stefanadis, et al., 2000, Stefanadis, et al., 1997). Other techniques such as the intima media thickness (IMT) can be used to assess the thickening of the carotid vascular wall, by external ultrasound or invasive ultrasound catheters to detect early stages of atherosclerosis or follow the evolution of plaques (Ubels, et al., 1999).

1.3.1.2 FA and arterial stiffness

- *Arterial stiffness as an indicator of CVD risk*

IMT, assessing structural rather than functional changes, is a well established marker of atherosclerotic burden and has prognostic value (Ubels, et al., 1999). Elevated PWV has been associated with the presence of several CVD risk factors such as age (Simonson and Nakagawa, 1960, Vaitkevicius, et al., 1993), hypercholesterolemia (Levenson, et al., 1992, Riggio, et al., 2010) and hypertension (Dahan, et al., 1990, Safar and London, 1987). A recent meta-analysis showed that aortic stiffness, as measured by

PWV is a strong predictor of CV events and all cause mortality, independent of classic CVD risk factors and other potential confounders, with a better predictability for subjects having higher CVD risk baseline (Vlachopoulos, et al., 2010). Radial and carotid AIx have been positively associated to the risk of CVD such as CHD, atherosclerosis, and stroke in some studies (Otsuka, et al., 2010, Qureshi, et al., 2007) but not all (Krantz, et al., 2011) and appears a weaker predictor of CVD risk than PWV (Van Trijp, et al., 2006). Arterial stiffness being an additional independent risk factor for CVD, strategies aimed at lowering arterial stiffness, including dietary interventions may be effective in reducing CVD risk. Studies to investigate the effect of dietary FA on such outcome are only at their beginning.

- *Evidence from human studies*

The effect of dietary FA on vascular function and BP has been recently extensively reviewed (Hall, 2009). Chronic dietary intervention studies suggest that n-3 PUFA and to a lesser extent MUFA, are beneficial for BP while saturated appear detrimental (Hall, 2009). However the effect of MUFA and SFA on arterial stiffness specifically has been poorly studied, acutely or chronically.

Acute effects: The effect of meal FA composition on post-prandial arterial stiffness remains uncertain. SFA (rich in stearic acid) was shown to impair stiffness index in healthy men in some studies (Newens, et al., 2011) but not all (Berry, et al., 2008). Recent evidence suggest that EPA- and DHA-enriched meals reduce arterial stiffness as measured in healthy males, as measured by DVP and PWA (Chong, et al., 2010, Hall, et al., 2008), but data is limited.

Chronic effects: In healthy subjects, a MUFA-enriched diet provided for 4 weeks did not improve arterial elasticity as measured by carotid and derived aortic pulse wave analysis compared to an isocaloric low fat /high carbohydrate diet (energetically equivalent) (Ashton, et al., 2000). In overweight subjects, PWV was improved by an 8 weeks weight loss programme with no adverse effect of SFA (Keogh, et al., 2008). In healthy subjects, a Mediterranean diet (8% SFA, 14% MUFA) had no differential effect on carotid distensibility (echo-tracking) compared to a Swedish diet (36% SFA, 12% MUFA) provided for 4 weeks (Ambring, et al., 2004). More recently, our group showed that 6 months of a high SFA and high MUFA dietary intervention had no differential effect on stiffness index (SI), as measured by DVP, although SI was improved with a low fat diet

compared to the high SFA and MUFA diets (Sanders TA, 2008). While the effect of SFA and MUFA on arterial stiffness remains uncertain, evidence is progressively accumulating regarding the effect of n-3 PUFA from fish oils. A recent meta-analysis including 10 randomised controlled trials concluded that n-3 PUFA had a beneficial effect on arterial stiffness and compliance, even after adjusting for BP, BMI and heart rate (Pase, et al., 2011). However it should be noted that this study considered all populations (including healthy, overweight and diabetics) and two types of measurements (PWV and arterial compliance). More interventions are needed to confirm these results and clarify their effect on different populations.

1.3.2 FA and endothelial function (EF) in humans

1.3.2.1 Assessment of EF

The deleterious effects of CVD risk factors (i.e. hypertension, hypercholesterolemia, diabetes, obesity, cigarette smoke) elicit a systemic inflammatory response. In blood vessel at both macro- and microvascular levels, the endothelium appears to be their major cellular target. Despite the multiplicity of the functions mediated by the endothelium, the assessment of EF in humans amounts to evaluating the degree of endothelium-dependent vasodilation, particularly NO-induced vasodilation. Peripheral arteries, including the brachial artery, respond to physical and chemical stimuli by adjusting vascular tone and regulating blood flow. Non invasive techniques are usually applied to the arm or the forearm and assess EF in the brachial artery or the resistance vessels of the upper arm.

- *Flow-mediated dilatation*

An increase in blood flow, provided by reactive hyperaemia (RH), leads to shear stress which stimulates endothelial NO release, inducing vasorelaxation (described earlier in Figure 1-5). The vasodilatory response of the brachial artery to shear stress is called flow mediated dilation (FMD) and is evaluated by the use of high resolution ultrasound. In this method, an ischemic cuff is placed either around the wrist or the upper arm and inflated for 5 min at a pressure of 50 mmHg higher than the SBP. The brachial artery is continuously monitored in the baseline state (1min), during cuff inflation (5min), and postcuff release (5min) (Sanders, et al., 2011). The maximal diameter is detected approximately 30-90s

after the cuff release (Corretti, et al., 2002). Most laboratories define FMD as the percentage change of the brachial artery diameter from baseline (before ischemia) to the maximal diameter. FMD is mainly endothelium-dependent (NO dependent) and inversely correlated to baseline arterial diameter. It should be noted that FMD responses in humans are not all equally NO dependent, and may involve other vasodilators, vessel wall structural factors (elastin, collagen) (Green, et al., 2011). Glyceryl trinitrate (GTN, NO donor) is often administered sublingually to isolate NO-dependent effects (Vogel, 2001).

- *Gauge-strain plethysmography: Forearm blood flow measurement*

In contrast with FMD that assess changes in diameter in the brachial (conduit) artery, gauge-strain plethysmography evaluates the changes in blood flow in the forearm (resistance vessels) in response to a 5 min RH. Forearme blood flow (FBF) is measured using a strain gauge attached to the upper part of the left arm and connected to a plethysmograhy device. A congesting cuff is placed on the upper arm and inflated to 40 mmHg for 7s in each 15s cycle to occlude venous outflow, using a rapid cuff inflator. A second cuff is placed on the wrist and inflated at 50 mmHg above SBP for 5 min to exclude the hand from the measurement. FBF is measured every 15s after the release of the ischaemia for 3 min. Forearm vascular resistance may be calculated as the mean BP divided by FBF (expressed as a mm Hg/ml/min per 100 ml of tissue of forearm volume) (Higashi and Yoshizumi, 2003).

- *Infusion of vasoactive agents*

The intrabrachial administration of vasoactive agents such as L-NMMA (eNOS inhibitor), acetylcholine (eNOS activator) or sodium nitroprusside (SNP, NO donor) may be combined to FMD or FBF measurements in order to specify the role of NO release in vasodilation (Higashi and Yoshizumi, 2003, Kooijman, et al., 2008, Mosca, et al., 2009). Similarly, changes in coronary diameter and coronary blood flow may be measured in response to intracoronary agonist infusion (Reriani, et al., 2010, Tousoulis, et al., 2005). Alternatively, techniques such as PWA and DVP described in 1.3.1 can be used in combination with vasodilators such as the β-2 adrenoceptor agonist salbutamol, administered by inhalation or intravenously (Donald, et al., 2006).

- *Circulating markers of EF*

While the techniques described above assess EF locally, the measurement of circulating biomarkers may allow the estimation of EF systemically. Several traditional biochemical markers have been measured in plasma as indicators of EF, such as NO metabolites (nitrites/nitrates), prostacyclin, soluble thrombomodulin, von Willebrand factor, or the tissue factor pathway inhibitor. In addition, novel biomarkers have emerged, such as ADMA, described earlier, as well as circulating progenitor cells and endothelial progenitor cells, representing the ability for vascular repair, as well as circulating endothelial cells and endothelial microparticles, representing the degree of endothelial damage (Li, et al., 2011).

EPCs are a subgroup of circulating progenitor cells (CPC) that are recruited from the bone marrow to repair the injured vasculature (See Figure 1-7:). They have been associated with a reduced CVD risk (O'Dunn-Orto, et al., 2012) and may serve as markers of EF because they represent a greater capacity for the endothelium to repair itself. Their number and capacity of differentiation correlates with FMD (Miura, et al., 2009) and their improvement upon endurance training in subjects at high CVD risk is associated with the improvement of FMD and NOx levels (Steiner, et al., 2005). As a transitory state between bone marrow cells and endothelial cells, endothelial progenitor cells (EPCs) express different markers throughout their differentiation. The lack of a specific marker, in addition to their rarity in peripheral blood (~ 0.002% of total peripheral mononuclear cells) (Peichev, et al., 2000), makes EPCs a challenging population to identify by flow cytometry. CD133 and CD34 are early hematopoietic stem cell markers expressed on hematopoietic stem and progenitor cells from human bone marrow (Andrews, et al., 1989, Yin, et al., 1997). EPC have been characterized by the co-expression of CD34, CD133 and vascular endothelial growth factor receptor-2 (VEGFR-2), also termed kinase insert domain receptor (KDR) or Flk-1 (Peichev, et al., 2000). However, while KDR is expressed all along their maturation, EPCs progressively lose CD133, and subsequently CD34, while they start expressing CD31, as expressed by mature endothelial cells (Hristov, et al., 2003).

EPC contrast with circulating endothelial cells (CEC), which include microparticles (MP), and are released from the endothelium when it is injured and are characteristic of ED (Erdbruegger, et al., 2010). CPC express early markers of differentiation such as CD133

and CD34, and EPC express markers of endothelial cells such as KDR in addition to the early markers.

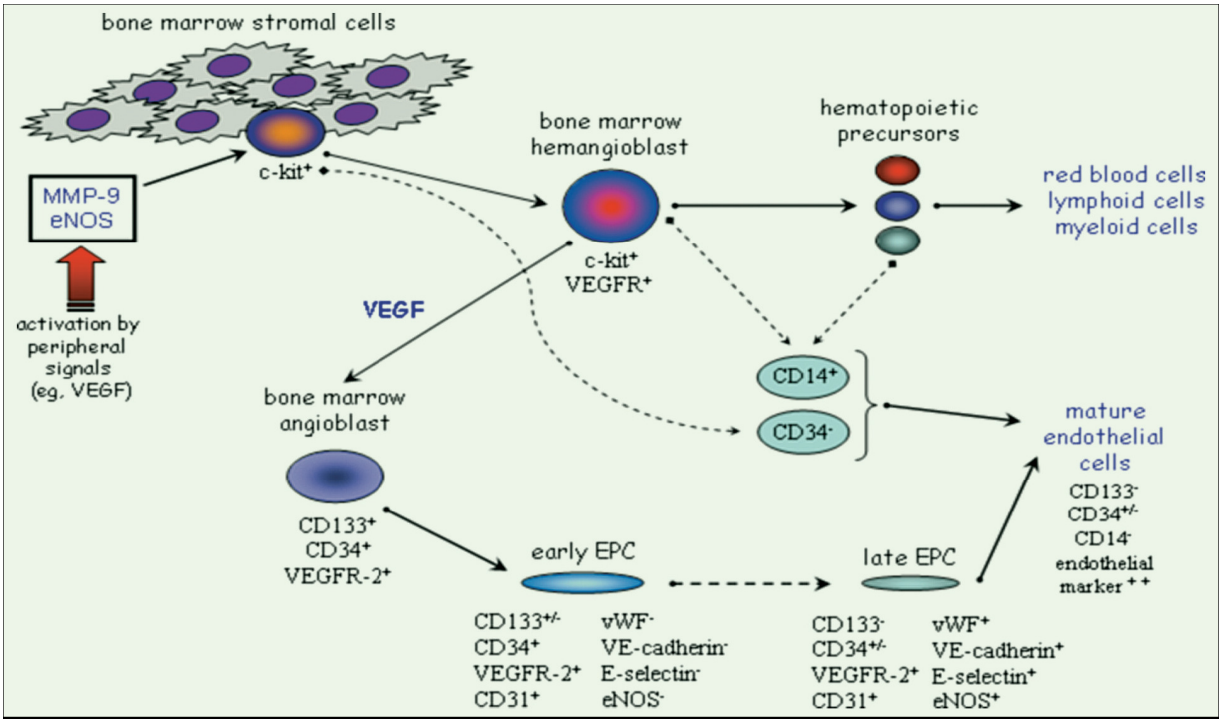

Figure 1-7: Mobilization, recruitment, and differentiation of human, bone marrow-derived angiogenic progenitor cells. (Hristov and Weber, 2004)

Vascular and endothelial injury leads to the activation of matrix metalloproteinase-9 (MMP-9), which promotes the transformation of membrane bound Kit-ligand to a soluble Kit-ligand in the bone marrow. This leads to the by detachment of early cKit+ progenitor cells and their movement from the stromal niche to the vascular zone of the bone marrow. VEGF plays an important role in EPC maturation by binding its receptor VEGFR-2 on the EPCs. Early EPCs (CD133+/CD34+/VEGFR-2+/CD14-) represent a small population with proliferative potential, capable to give rise to late endothelial outgrowth. Cells of myeloid origin (CD14+) may also trans-differentiate into endothelial cells and secret angiogenic factors, but their proliferative potential is limited. Mesenchymal CD34- progenitor cells can also generate endothelial cells; however, these cells showed in vivo reduced functional activity and incorporation into neo-vessels adapted from (Hristov and Weber, 2004).

1.3.2.2 FA and EF: Evidence from human studies

FMD is an independent predictor of CV events in chronic heart failure (Meyer, et al., 2005), peripheral and coronary artery disease (Brevetti, et al., 2003, Chan, et al., 2003) and has emerged as a potential prognostic tool (Green, et al., 2011). FMD is the most commonly used technique, and encounters a growing interest in dietary interventions, especially with fish oils. Although there is growing evidence from *in vitro* studies that n-3 PUFA improve EF (Deanfield, et al., 2007, IoM, 2005), the effect remains uncertain in humans. Some epidemiological studies suggest that PUFA, especially n-3 PUFA are beneficial for EF while SFA appear detrimental (Augoustides, et al., 2007, Harris, 2008, Maa, et al., 2007, Mozaffarian, et al., 2005, Yli-Jama, et al., 2002). However recent data showed no correlation between FMD with neither phosholipid EPA and DHA nor fish intake in healthy subjects (Hjelte, et al., 2005).

Numerous studies have investigated the acute effect of saturated, monounsaturated and n-6 PUFA on EF but findings remain conflicting (Vafeiadou, et al., 2012). Recent data suggest that n-3 PUFA enriched meals improve post-prandial EF in type 2 diabetics (Hilpert, et al., 2007, West, et al., 2005) but evidence remains limited.

Several chronic studies have reported a beneficial effect of n-3 PUFA on circulating markers of endothelial activation in healthy subjects, including ICAM-1, VCAM-1, E or P-selectin (Bennett and Horrobin, 2000, Horrobin, 2000, MacDonell, et al., 2000), although this is not always the case (Puri, et al., 2000). Markers of endothelial activation also appear to be improved in patients with type 2 diabetes or dyslipidaemia while results are conflicting in subjects with CVD (Ueshima, et al., 2007). However, measurement of vasodilatory function by FMD and FBF were improved by EPA and/or DHA in several studies in patients with CVD (Schiano, et al., 2008), type 2 diabetes (Rizza, et al., 2009, Stirban, et al., 2010) or dyslipidaemia (Goodfellow, et al., 2000, Mori, et al., 2000). Regarding the effect of SFA and MUFA, evidence from randomised controlled trials is even more limited and conflictive (Hall, 2009). A randomised cross-over trial showed that a 3-week high-SFA diet led to an impairment of EF compared with high-PUFA, -MUFA or -carbohydrate diets (Rajendran, et al., 2007). The same study showed a decrease of P-selectin levels with high-MUFA and -PUFA diets compared with the high-SFA diet. More recently, another randomised cross-over trial (4 weeks intervention) showed an improvement of EF upon a high-MUFA diet compared to a high SFA diet or a low fat diet

enriched with ALA (Strauss, et al., 2007). ED is closely related to platelet dysfunction and NO plays a crucial role in platelet activation, including P-selectin expression and platelet-monocyte aggregation (Gkaliagkousi, et al., 2009). Platelet-monocyte aggregates were shown to be reduced by fish consumption for 4 weeks in healthy young males (Din, et al., 2008).

However, it should be noted that beyond the impairment of vasodilation, ED also involves the imbalance between other endothelium dependent processes (Cf. 1.2.5) and little work has been done to relate the different EF markers to one other in disease state (Celermajer, 2008).

1.3.3 FA and microvascular function

ED is a globalised systemic disease characterised by an impairment of vasodilation and constriction, and microvessel structural remodelling (arterioles and capillaries). The microcirculation (which encompasses a vast endothelial surface area) may not only serve as a target for the detrimental effects of CVD risk factors but has also been implicated in the initiation and/or progression of large vessel disease (Stokes and Granger, 2005, Suematsu, et al., 2002). Most non invasive measurement techniques described earlier to assess vascular function in humans, assess the vessel wall stiffness or EF in large arteries; but the estimation of microvascular function, systemically or locally, may also be of great interest. The microcirculation can be examined over large areas of skin by laser Doppler imagery, or more locally by laser Doppler flowmetry. Changes in the vasodilation and constriction of skin microvessels may be induced by RH, whole body heating or cooling, local heating or cooling, or by the application of vasoactive agents non invasively (iontophoresis) or using minimally invasive techniques such as intradermal microdialysis (Holowatz, et al., 2008).

Skin and sublingual microcirculation are the only vascular beds that are accessible for clinical assessment. New techniques of videomicroscopic examinations have emerged and could be of great interest for diagnosis and/or research. Capillaries are only made of one layer of endothelium and investigating their structure and/or function can provide useful information on EF. Capillary rarefaction has been associated to CVD risk factors such as hypertension (Antonios, et al., 1999), smoking (Debbabi, et al., 2006) and obesity

(Sioen, et al., 2007). The cutaneous circulation has emerged as an accessible and representative vascular bed to look at microvascular dysfunction (Holowatz, et al., 2008), although its extrapolation to systemic and peripheral microvascular function remains controversial (Varenne, et al., 2006, Weber, 2006). Nonetheless, capillary density, i.e. the number of capillaries per skin area, can be easily assessed in the finger using a laser camera, and has shown inverse correlation with hypertension (Sioen, et al., 2007). Furthermore, video facilities allow real-time imaging which can provide information on the morphology and velocity of red blood cells (RBCV) (Awan, et al., 2010). RBCV is more often measured by Doppler (Bilau, et al., 2007, Sioen, et al., 2007). In addition several imaging techniques have been developed in order to measure RBCV at the nailfold, where the capillaries are horizontal. Those techniques include the frame to frame (Butti, et al., 1975, Mugii, et al., 2009), the flying spot (Gasser and Buhler, 1992, Mahler, et al., 1987, Paul, 1990) or the cross-correlation (Fagrell, et al., 1977, Intaglietta and Tompkins, 1987, Mawson and Shore, 1998) techniques.

Despite the development of these techniques, microvascular function has been poorly investigated in nutritional intervention. Some studies suggest that LC *n*-3 PUFA enriched meals may improve post-prandial microvascular reactivity through endothelium-independent mechanisms (Armah, et al., 2008). However there is, to our knowledge, no data regarding the chronic effect of fats on capillary structure and function.

1.4 Aim and objectives

1.4.1 Aim

The aim of this thesis is to further the understanding of the effect of different dietary FA on EF in humans, *in vivo* and *in vitro*. We hypothesized that the nature and proportion of FA differentially affects EF *in vivo* and *in vitro*.

1.4.2 Objectives

The objectives of our *in vivo* work were to investigate the differential effect of a 6-week EPA versus DHAsupplementation in healthy young males compared to placebo on:

- markers of endothelial function (EPC, NOx)

- macrovascular function (PWA, DVP)

- microvascular function (capillary density)

- related markers of CVD risk (such as markers of oxidative stress, lipid profiles)

The objective of our *in vitro* work was to test the effect of various FA profiles, characteristic of physiological NEFA profiles observed after different dietary patterns, on EF, as measured by NO and prostacyclin production, in human microvascular endothelial cells.

Chapter 2 The EPA and DHA trial - a randomised controlled dietary intervention study: Materials and Methods

The EPA and DHA trial was a single-blind, randomised, parallel study designed to test the effects of EPA and DHA supplementation (3g/d, 6 weeks) vs placebo (olive oil) on EF in healthy young men. It was carried out by the author and Dr Aseel Alsaleh. Both were in charge of the recruiting the participants and providing them with all information at screening. Dr Aseel Alsaleh was responsible for the pulse wave analysis, the digital volume pulse, the body composition measurements and blood handling. The author was responsible for scheduling the participants, the ambulatory blood pressure (ABP) monitoring and giving the associated instructions, the microvascular measurements (capillaroscopy), phlebotomy and the treatment of fresh blood samples for flow cytometry analysis. The serum adiponectin, HOMA-IR and plasma lipid results have already been reported in the thesis of Dr Aseel Alsaleh (AlSaleh, 2011).

2.1 Materials

2.1.1 List of equipment

- Centrifuge Jouan CR4.12 for blood handling (DJB Labcare Ltd, Buckinghamshire, UK)
- Calibrated automated blood pressure monitor (Omron 705IT, Omron Healthcare Europe B.V.)
- Bioelectrical impedance analysis equipment (BC-418 MA, Tanita UK Ltd, Middlesex, UK)
- Digital Volume Pulse (DVP; PulseTrace PCA 2, Micro Medical Ltd)
- Pulse Wave Analysis (PWA; SphygmoCor Px, AtCor Medical Pty Limited)
- CapiScope Capillaroscopy System (KK Research Technology Ltd, Devon, UK)
- FC500-Beckman Coulter flow cytometer (Beckman & Coulter Ltd, UK)
- 5 Ambulatory blood pressure monitors (TM-2430, A&D Instrument Ltd, Abingdon, U.K.)

- Eppendorff concentrator 5301 (Eppendorf UK Ltd, Cambridge, UK)
- Agilent 6890 Gas Chromatograph (Agilent Technologies UK Ltd, Wockingham, UK)
- Centrifuge Beckman GS-6R (Beckman & Coulter Ltd, UK)
- Eppendorff concentrator 5301 (Eppendorf UK Ltd, Cambridge, UK)
- GC-MS instrumentation with negative chemical ionization capability: Agilent Technologies 6890N network Gas Chromatograph system equipped with 7683 series autoinjector, PTV (Gerstel) Inlet and 5673 inert mass selective detector with chemical ionization module
- D Data/Control System: Agilent Enhanced MSD Chem Station running on Windows XP Professional
- Sample processing station (Argonaut Vacmaster®-20, Biotage (UK) Ltd, UK)
- PTI-11 digital pH metre (Paper testing instruments GmbH, Austria) and pH electrode (Russell Ph Ltd, Auchtermuchty, UK)
- 8-Isoprostane affinity columns (Cayman Chemical Europe cat. no. 416358, VWR International Ltd, UK)
- Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane, 10kDa (cat.no. UFC501096, Millipore Ltd., UK)
- Partisil® absorption thin layer chromatography plates LK6D (cat.no. 4865-821, Whatman international Ltd, Kent, UK)
- Venous blood collection tubes:
    - 4.5 mL EDTA lavender tubes (Becton Dickinson; cat.no. 367654, UK)
    - 4 mL fluoride/oxalate (FX) grey tubes (Becton Dickinson; cat.no. 367922, UK)
    - 8.5 mL gold tubes with clot activator and gel for serum separation (Becton Dickinson; cat.no. 367953, UK)
    - 4.5 mL citrated light blue tubes (Becton Dickinson; cat. no. 369714, UK)
    - 4 mL lithium heparin (LH) green tubes (Becton Dickinson; cat. no. 367884, UK)

2.1.2 Solvents and acids

- Methanol (HPLC grade, Fisher Scientific, UK)
- Chloroform (BDH AnalaR, VWR International Ltd., UK), 50mg BHT/L

- Isopropanol (BDH AnalaR, VWR International Ltd., UK), 50mg BHT/L
- Hexane (HPLC grade, Fisher Scientific, UK)
- Absolute Ethanol (Sigma-Aldrich, Dorset, UK)
- Acetone (HPLC grade, Fisher Scientific, UK)
- Isooctane (Puriss grade Fluka-Chemika, Sigma-Aldrich, Dorset, UK)
- Diethylether (BDH AnalaR, VWR International Ltd., UK)
- Toluene (BDH AnalaR, VWR International Ltd., UK)
- Formic acid (Fluka-Chemika, Sigma-Aldrich, Dorset, UK)
- Acetyl chloride (Sigma-Aldrich, Dorset, UK)

2.1.3 Chemicals, reagents and kits

- Butylated hydroxytoluene (BHT, Sigma-Aldrich cat.no. B1378, Dorset, UK): 5 mM stock solution in ethanol
- Sodium hydrogen carbonate ($NaHCO_3$, BDH AnalaR, VWR International Ltd., UK)
- Indomethacin (Sigma-Aldrich; cat.no. I7378, Dorset, UK): 2 mM in 5% w/v aqueous $NaHCO_3$
- Ethylenediaminetetraacetic acid (EDTA, BDH cat.no. 100935V, VWR International Ltd., UK)
- Sodium methoxide ($NaOCH_3$) 0.5N in anhydrous methanol (Sigma-Aldrich, cat.no. 156256, Dorset, UK)
- Calcium chloride ($CaCl_2$, BDH AnalaR, VWR International Ltd., UK)
- Potassium carbonate (K_2CO_3, BDH AnalaR, VWR International Ltd., UK) 6 % (w/v) in UltraPure water
- Pentadecanoic acid (C15:0, internal standard for NEFA,50 mg/mL in $CHCl_3$:MetOH 2:1 v/v) (~99% by capillary GC, Sigma-Aldrich, cat no. P6125-5G, Dorset, UK)
- Sodium chloride (NaCl, BDH AnalaR, VWR International Ltd., UK)
- Potassium chloride (KCl, BDH AnalaR, VWR International Ltd., UK)
- Magnesium sulphate, hydrated ($MgSO_4$, $7H_20$, Fisons-Fisher Scientific, Loughborough, UK)
- Hepes (Sigma-Aldrich cat.no. H0891, Dorset, UK)
- iso-8-Prostaglandin $F_{2\alpha}$ (iso-8-$PF_{2\alpha}$ 1mg, Cayman Chemical Europe cat.no. 16350-1, VWR International Ltd, UK): 10µg/mL in ethanol (standard solution)

- iso-8-Prostaglandin $F_{2\alpha}$ -17,18,19,20 – D_4 (iso-8-$PF_{2\alpha}$-D_4 25µg/250µl Methyl Acetate, Cayman Chemical Europe cat.no. 316350, VWR International Ltd, UK): 1µg/mL in ethanol (internal standard solution)
- Anhydrous potassium hydroxide pellets (KOH, BDH AnalaR, VWR International Ltd., UK)
- Dipotassium hydrogen ortho phosphate (K_2HPO_4, BDH AnalaR, VWR International Ltd., UK)
- Potassium diHydrogen ortho Phosphate (KH_2PO_4, BDH AnalaR, VWR International Ltd., UK)
- Acetic acid (BDH AnalaR, VWR International Ltd., UK)
- Sodium Azide (NaN_3, BDH AnalaR, VWR International Ltd., UK)
- N,N-Diisopropylethylamine (DIPEA, Sigma-Aldrich, cat.no. 387649, Dorset, UK)
- α -Bromo-2,3,4,5,6-pentafluorotoluene (PFBBr, Sigma-Aldrich, cat.no. 101052, Dorset, UK)
- N,O-bis(Trimethylsilyl)-trifluoroacetamide (BSTFA, Pierce® Thermo Scientific, UK)
- Total Nitric Oxide and Nitrite/Nitrate Assay kit (cat.no. KGE001, R&D Systems Europe Ltd, UK)

2.1.4 Solutions and buffers

- Saline 0.89% NaCl, 40mg EDTA/L
- Phosphate buffered saline (Sigma-Aldrich, cat.no. P4417, Dorset, UK)
- Erythrolyse red blood cell lysing buffer 10X (cat.no. BUF04B, AbD Serotec, Oxfordshire, UK)
- IOTest® 3 Fixative Solution, 100 - 200 Tests, ready to use (cat.no. A07800, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Optilyse® C lysis solution, 200 tests, ready to use (cat.no. A11895, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Hepes Buffer Saline (HBS): NaCL 150 mM, KCL 5 mM, $MgSO_4.7H_2O$ 1 mM, Hepes 10 mM.
- Isoprostane standard solution: iso-8-$PF_{2\alpha}$ 10µg/mL in ethanol
- Isoprostane internal standard solution: iso-8-$PF_{2\alpha}$-D_4 1µg/mL in ethanol

- Affinity column buffer solution, 0.1 M PBS pH 7.4 : 13.3g K_2HPO_4, 3.22g KH_2PO_4, 0.5g NaN_3, 29.2g NaCl in 1L UltraPure water.
- Affinity column elution solution: Ethanol 95% v/v in Ultrapure water
- Alkalinisation solution: 15% w/v potassium hydroxide in UltraPure water
- 1M potassium dihydrogen ortho phosphate in UltraPure water
- DIPEA solution: 10% v/v in acetone
- PFBBr solution: 20% v/v in acetone.

2.1.5 Antibodies (Flow cytometry)

- Mouse Anti-Human IgG1-FITC (cat.no. IM0639, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Mouse Anti-Human IgG1-PE (cat.no. IM0670, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Mouse Anti-Human IgG1-APC (cat.no. IM2475, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- IgG1, Kappa from murine myeloma, clone MOPC31 (Sigma-Aldrich, cat.no. M9035, Dorset, UK)
- Monoclonal anti-human P-Selectin/CD62P antibody, clone 9E1 (cat.no. BBA30, R&D Systems Europe Ltd., UK)
- Mouse Anti-Human CD31 (PECAM-1)-FITC (cat.no. IM1431, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Mouse Anti-Human CD45-PE (cat.no. IM2078, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Mouse Anti-Human CD34-PE (cat.no. A0776, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Mouse Anti-Human CD34-FITC (cat.no. IM1870, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Monoclonal anti-human VEGF R2/KDR-APC (cat.no. FAB357A, R&D Systems Europe Ltd., UK)
- Mouse Anti-Human CD133/1 (AC133)- PE (cat.no. 130-080-801, Miltenyi Biotec Ltd., UK)

- Mouse Anti-Human CD14-FITC (cat.no. IM0645, Beckman & Coulter UK Ltd, Buckinghamshire, UK)
- Mouse Anti-Human CD42b-PE (cat.no. IM1417, Beckman & Coulter UK Ltd, Buckinghamshire, UK)

2.1.6 Supplements

- 500mg EPA-rich oil softgel capsules (Incromega EPA500TG SR, Croda Chemicals Europe Ltd, Goole, UK)
- 1000mg DHA-rich oil soft gel capsules (Incromega DHA500TG SR soft gel capsules, Croda Chemicals Europe Ltd, Goole, UK)
- 1000mg olive oil soft gel capsules (Croda Chemicals Europe Ltd, Goole, UK)

2.2 Methods and methodology development

2.2.1 Participants

Healthy males, non smokers, aged between 18 and 45 years old were recruited. The participants were excluded according to the following specified criteria:

- Reported history of CVD (myocardial infarction, angina, venous thrombosis, stroke, dyslipidemia), diabetes (or fasting glucose ≥ 6.1 mmol/L), cancer, kidney, liver or bowel disease.
- Presence of gastrointestinal disorder or use of drug, which is likely to alter gastrointestinal motility or nutrient absorption.
- Current smokers; history of substance abuse or alcoholism (previous weekly alcohol intake >60 units/week); current self-reported weekly alcohol intake exceeding 28 units
- Recent use of hypolipidaemic, antihypertensive, antiplatelet or antithrombotic mediations
- Platelet count above or below the normal range or any history indicative of a congenital or acquired platelet or haemostatic defect.
- Allergy or intolerance to any component of study capsules
- Unwilling to restrict consumption of any source of fish oil for the length of the study
- Subjects reporting consumption of >1 portion oily fish per week
- Weight change of >3 kg in preceding 2 months; BMI <18 and >32 kg/m2

- Blood pressure>160/90 mmHg
- Fasting blood cholesterol > 6.5 mmol/L; fasting triacylglycerol concentrations > 2.0 mmol/L

2.2.2 Recruitment methodology

Participants were recruited through internal email circulars (0) and posters (Appendix II) among King's College London students and staff, and fitness centre users in the London Bridge area. The methodology of recruitment is outlined in Figure 2-1. Volunteers expressing interest were initially sent an information sheet (Appendix III) and a booklet (Appendix IV) summarising the study by e-mail. Those who responded were initially interviewed via a questionnaire over the phone (Appendix V), to assess whether they were suitable for the study. Volunteers who were eligible were then invited to attend a screening session during which blood pressure, height and weight, percentage body fat and waist and hip circumference were measured. At the end of the screening visit (2.2.4), a small blood sample was taken for a full blood count, lipid profile, liver function tests and fasting glucose concentrations. Volunteers who met the inclusion criteria described in 2.2.1 were enrolled in the study.

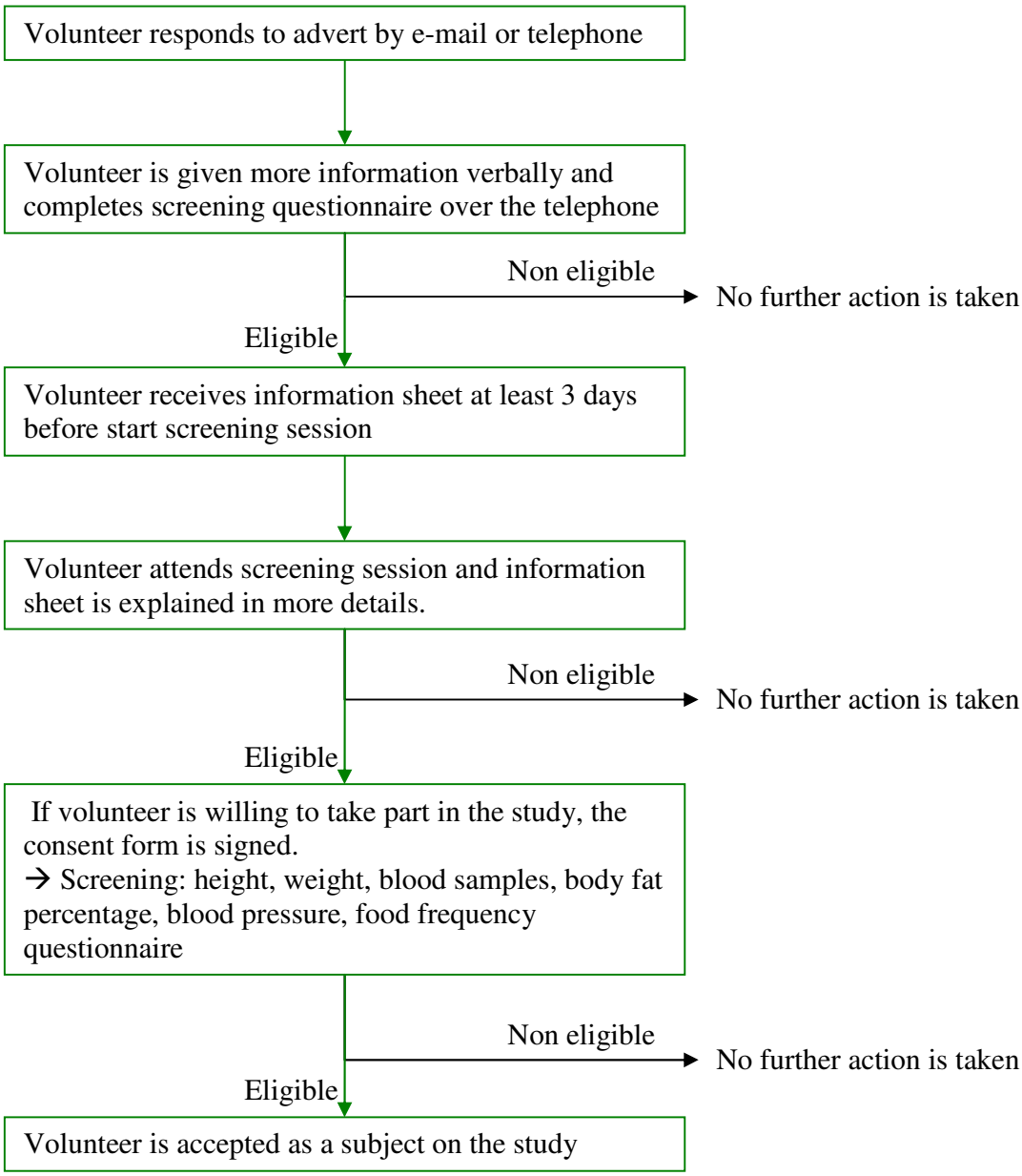

Figure 2-1: Methodology of recruitment - Outline

2.2.3 Study design.

A randomised single-blind parallel design was used to test the effects of formulations of DHA or EPA versus placebo on primary and secondary outcomes as outlined in Figure 2-2.

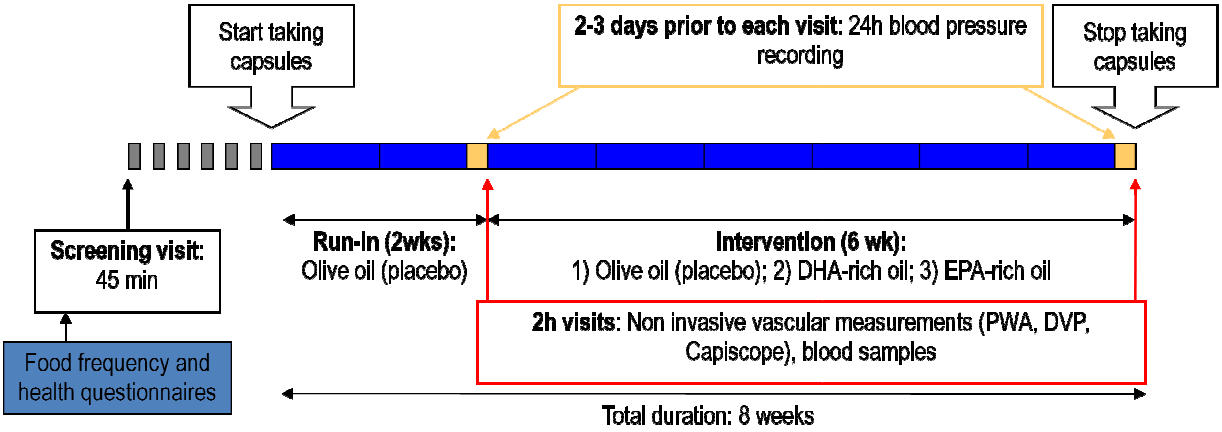

Figure 2-2: Outline of study protocol

The study protocol was approved 29th February 2008, with substantial amendments approved in April 2009, by the Bromley Research Ethics Committee (ref. 08/H0805/2), now called NRES Committee London-Bromley. A parallel design was chosen because of the duration of the intervention and the length of time taken for cell membrane fatty acid composition to return to baseline proportions following cessation of supplementation (approximately 2-4 months) (Brown, et al., 1991, Cao, et al., 2006). A 6-wk treatment period was chosen since previous studies have indicated that this is a sufficient length of time for EPA and/or DHA to induce significant changes in several CVD risk factors such as TAG levels (Mori, et al., 2000, Woodman, et al., 2002) and BP (Mori, et al., 1999, Woodman, et al., 2002).

In order to standardise intakes prior to the beginning of the study, the intervention started with a two-week run-in period during which participants were taking placebo capsules (an oleic acid-rich triacylglycerol, refined olive oil, BP specification). The participants were then allocated to treatment using a randomisation website (www.randomization.com), and consumed either olive oil (placebo), DHA-rich oil or EPA-rich oil for 6 weeks. Thus the total study time was 8 weeks, during which the subject was asked to avoid medications and the consumption of oily fish, fish oil, fatty acid or other dietary supplements. A list of oily fish – rich in EPA and DHA – to avoid, as well as a list of lean white fish (allowed) was provided (Appendix VI).

♦ *Capsules*

EPA- and DHA-rich oils, as well as olive oil were provided in the form of triglycerides in soft gel capsules. Refined olive oil and DHA-rich oil were supplied as 1 g capsules while EPA-rich oil was supplied as 500 mg capsules. Both EPA- and DHA-rich oils were blended with refined BP specification olive oil, with a content of at least 65% of total *n*-3 PUFA, including a minimum of 500 mg of EPA and DHA, respectively. The typical content of fatty acids in EPA- and DHA-rich oil was provided by the manufacturer (Appendix VII, VII. A): EPA-rich oils typically contain 58.4% and DHA-rich oils 57.5%. Calculations were based on the FA composition described by the manufacturer, to reach a consumption of ~3 g/d of EPA or DHA. The participants were asked to consume 5 g of supplement a day, *i.e.* 5 capsules a day in the placebo and DHA group (1g capsules), while the EPA group received 10 a day (500 mg capsules). The composition of the capsules was later analysed by GC in our department and confirmed similar composition (Appendix VII, VII. B), EPA and DHA reaching ~3.1 and 2.9 g/d, respectively. Precisely, subjects in the EPA group typically consumed ~3098 mg EPA + 103 mg DPA + 700 mg DHA per day, while the DHA group consumed 2865 mg DHA + 167 mg DPA + 517 mg EPA per day (Table 2-1)

	Capsules FA content, g/100g		FA intake (mg/d)	
Fatty acid	DHA-rich oil	EPA-rich oil	DHA-rich oil	EPA-rich oil
EPA	10.3	62.0	517	3098
DPA	3.3	2.1	167	103
DHA	57.3	14.1	2865	705

Table 2-1. EPA and DHA composition of EPA- and DHA- rich oils and resulting intake for the consumption of 5g of supplement a day.

For each period, the participants were provided with the equivalent of exactly 3 extra days in addition to the amount necessary for the run-in period (two weeks) and the intervention (six weeks) to ensure they had enough in case of loss or slight amendment of schedule. They were asked to bring the remaining capsules in the end of each period and capsule count was used as an indicator of compliance.

2.2.4 Screening visit

Participants arrived between 8.30 am and 1 pm (staggered at 45 minute intervals). On arrival to the metabolic unit of KCL, the participants were briefed on what the study involves and were provided with a hard copy of the 'information sheet for participants' and booklet previously sent by e-mail (Appendix IV, Appendix V). The participants were then required to sign the consent form (0).

After consent was obtained, the participants underwent a brief medical examination to measure blood pressure, waist and hip circumference, weight, height, and body composition using bioelectrical impedance (% body fat). Participants were excluded at this stage if any of their physical measurements did not fall within the prespecified limits described in 2.2.1. Standard physical measurement procedures are detailed in Appendix IX. All required information was documented on a standard screening record sheet (Appendix X).

A fasting blood sample was taken (16.5 ml) for the analysis of fasting glucose and plasma lipid concentrations (total, HDL and LDL cholesterol and TAG), liver function, and full blood count tests. Blood for haematology was collected into EDTA containing tubes, for plasma glucose into FX tubes, and for serum lipids and liver function into tubes containing clot activator and gel for serum separation. All tubes, apart from the EDTA tube (whole blood), were then centrifuged at 1300 g for 15 min at 2-4ºC and samples were dispatched by motorcycle courier on the day of collection to an accredited clinical pathology laboratory at King's College Hospital for same-day analysis (Clinical Pathology Accreditation Ltd. (CPA) laboratory (CPA 1245); Head: Dr Roy Sherwood). Participants were excluded at this stage if fasting blood samples were found to have abnormal glucose or lipid levels, liver function or haematology. The standard blood spinning protocol is described in Appendix XI

Lipids were measured via enzymatic assays and analysed on the ADVIA 2400 automated chemistry analyser (Siemens Healthcare Diagnostics, Surrey, UK). Detailed protocols are described in Appendix XII, Appendix XIII and Appendix XIV. TAG were hydrolysed into glycerol (lipase), which was then converted into glycerol-3-phosphate. The latter was then oxidised (glycerol-3-phosphate oxidase) to give hydrogen peroxide (H_2O_2), which yields a coloured pigment in the presence of peroxidase. Total cholesterol esters

were hydrolysed (cholesterolesterase) into free cholesterols, which were in turn oxidised to generate H_2O_2 that combines with phenol and 4-aminophenazone to give a red dye. The HDL method was a two step procedure described in Appendix XIV. All non HDL was converted into H_2O_2 through the choleterol esterase / cholesterol oxidase system in absence of detergent, which protects the HDL from the enzymes. H_2O_2 was removed by the action of catalase and the procedure was repeated in the presence of detergent and sodium azide (catalase inhibitor). The remaining HDL produced H_2O_2 which reacts with 4-amino-antipyrine to produce a blue purple compound. LDL cholesterol was calculated using the Friedewald formula (LDL = total cholesterol – HDL cholesterol – TAG / 2.2; in mmol/L) (Friedewald, et al., 1972).

Plasma glucose was determined by the hexokinase and glucose-6-phosphate dehydrogenase (G6PD) method (Appendix XV) on the ADVIA 2400 automated chemistry analyser (Siemens Healthcare Diagnostics, Surrey, UK). Glucose was phosphorylated into glucose-6-phosphate, which was then oxidised into 6-phosphogluconate. The $NADH+H^+$ produced by the last reaction in an increase of absorbance proportional to the glucose concentration in the plasma sample.

Full blood counts were analysed by routine chemistry using the ADVIA 2120 automated chemistry analyser (Siemens Healthcare Diagnostics, Surrey, UK). The method relies on four principles described in Appendix XVI The hydrodynamic focusing is used to produce a single stream of cells for flow cytometry analysis. The peroxidise methods differentiate the populations of white cells. The ‘baso’ method consists in lysing red blood cells, platelets and all white cells except the basophils. The cyanide free haemoglobin detection is used to quantify haemoglobin released by the red blood cells after lysis. The nucleated red blood cells are identified by their nuclear size measured in the peroxide channel and their nuclear density in the ‘baso’ channel.

Assays for liver function were carried out using the ADVIA 2400 automated chemistry analyser (Siemens Healthcare Diagnostics, Surrey, UK). Total protein was measured by the Biuret reaction (Appendix XVII), albumin by bromocresol green (Appendix XVIII), bilirubin by vanadate oxidation (Appendix XIX), aspartate aminotransferase using aspartate to glutamate conversion monitored by UV (Appendix XX), alkaline phosphatase using p-nitrophenol phosphate as substrate (Appendix XXI), and

gamma glutamyl transpeptidase using γ-glutamyl-4 nitroanilide as a substrate (Appendix XXII).

Finally the participants were requested to select an 8-wk period during which they were able to take supplements and attend the metabolic unit for 2 x 2-hour visit after 2 and 8 wk of intervention. A light breakfast was provided to the participants before leaving the screening session.

2.2.5 The study days

Two to 3 days prior to each study visit, participants were asked to record their blood pressure for 24 h. Thus, they were asked to attend the metabolic unit the week before their visit in order to collect the ABP monitor. This short visit lasted 20 to 30 min, during which the subject was shown how to use the ABP monitor and provided with a diary card where he had to record his activity during the 24 h BP monitoring, as well as written instructions (Appendix XXIII, Appendix XXIV). The subject was asked to bring back the ABP monitor and urine, as well as the diary card on the day of the visit.

Prior to each visit, the participants were contacted to ask them to avoid certain food/drinks and activities that may affect vascular function and BP. They had to avoid drinking alcohol, caffeine (from midday the previous day) and taking part in any strenuous exercise at any time during the day preceding their visit. They were asked to fast overnight and instructed to avoid eating or drinking anything, except water, for 12 h before the time of their scheduled study visit. They were also asked to consume a low fat meal as their evening meal. A reminder e-mail was sent out on the morning before the study day summarising the instructions (Appendix XXV).

One to two participants were scheduled between 8 am and 1 pm depending on their availability. On arrival, the participant was provided with water to avoid dehydration. The questionnaire subject record sheet (Appendix XXVI) was completed along the 2 h visit. The ABP monitor and diary card were collected. Seated BP and HR, as well as weight and body composition were then measured. The participant was then asked to rest quietly in a supine position for 15 min prior to the start of vascular measurements. The room temperature was recorded at that time (baseline: 24.17 +/- 0.50; 6 wks: 24.23 +/- 0.39). The programme of a typical study day is outline in Figure 2-3.

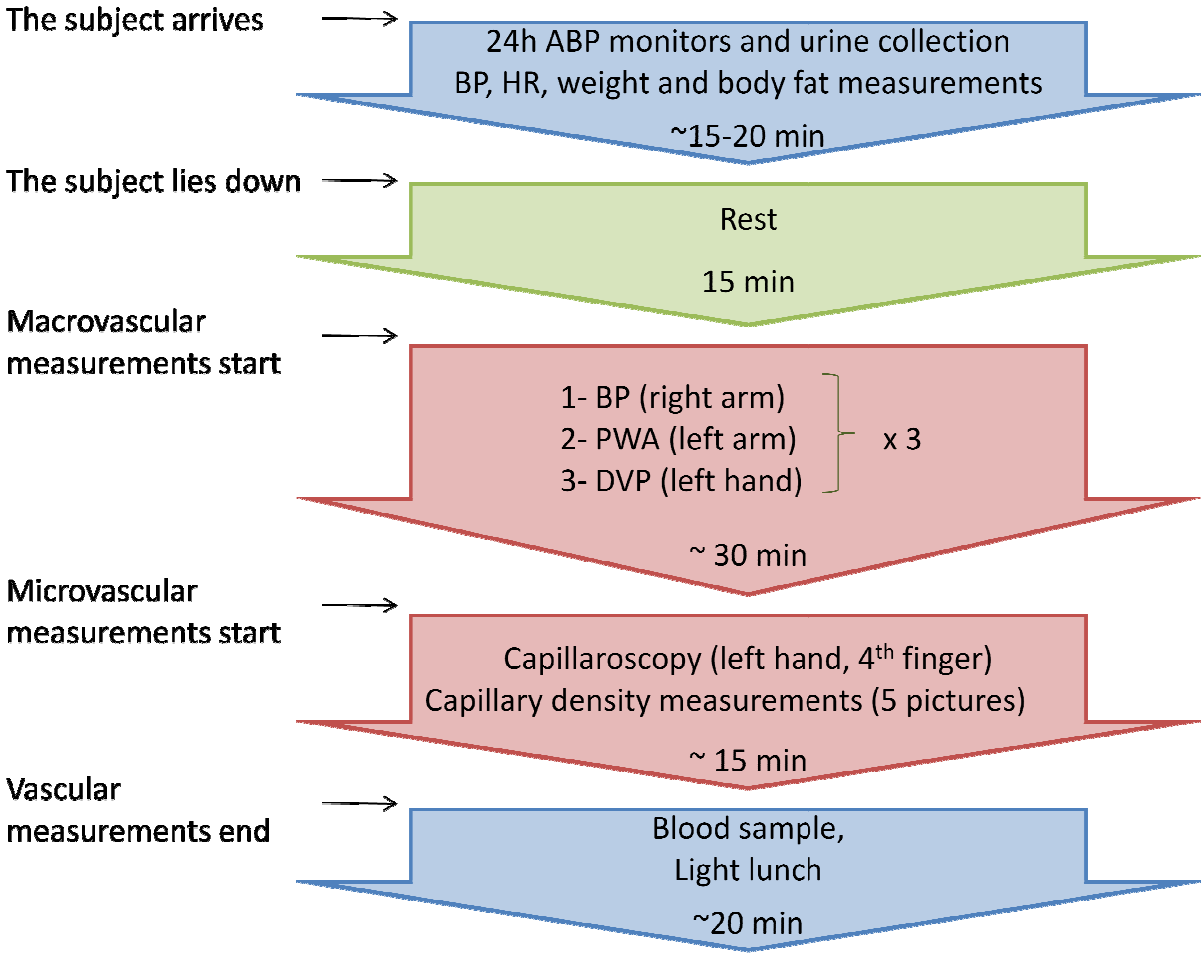

Figure 2-3. Outline of a study day

BP, Blood pressure; ABP, ambulatory BP; HR, heart rate; PWA, pulse wave analysis; DVP, digital pulse volume analysis

- *Vascular measurements (overview)*

PWA and DVP analysis were performed in order to measure central and peripheral arterial stiffness and pulse wave reflection. Capillary density was measured to assess potential structural changes in the microvasculature (finger capillaries). All the non-invasive vascular measurements (PWA, DVP, capillaroscopy) were made in the supine position and are further described in 2.2.12. They were carried out on the left arm, while BP was measured on the right arm.

Peripheral arterial tone was measured by digital volume pulse (DVP) using the forefinger pulse and pulse wave analysis (PWA) using the radial pulse, according to

extensive recommendations (Van Bortel, et al., 2002). Supine blood pressure was measured concurrently. These vascular measures were made in triplicate, according to the following sequence: (1) a single DVP measure on the left index finger, followed by (2) a blood pressure measurement of the right brachial artery, and finally (3) a PWA carried out on the left radial artery. When the finger pulse could not be detected by DVP in the index finger, the probe was changed to the thumb (same hand), which may give a stronger signal. If the signal was still too weak (due to the low temperature of the skin), a heating blanket was used to warm up the subject's hand. The temperature of the room was recorded in the end of the last DVP measurement and remained constant at 24.20 ˚C (±0.44). For both DVP and PWA analysis, the average of 3 measurements was taken, unless % COV of the 3 recordings was >15%, in which case the average of the 2 closest was taken. The measurements were made by Miss Aseel Alsaleh and the data entry and analysis were carried out by both Aseel and the author.

Next the subject was asked to place his left fourth finger in the finger device underneath the Capiscope camera. Five pictures were taken at the distal (terminal) phalanx, away from the nail fold, where capillaries are perpendicular to the skin and allow capillary density measurements.

- *Venepuncture and blood handling*

After the vascular measurements, the participant was advised again to drink water before the venepuncture. Blood was collected by the author according to the blood collection described in Appendix XXVI and Appendix XXVIII. The tourniquet was released on the 7th tube (EPC, Erythrocyte lipids) in order to ensure a steady flow for blood collection for prostacyclin and PMA measurement. The order of blood draw and the outcomes measured are outlined in Table 2-2.

Order of draw	Tube	Outcome measured	Comment
1	8.5 mL Serum (gold)	Adiponectin, CRP, Resisitin	Used by Aseel Alsaleh
2	8.5 mL Serum (gold)	FLIP, Nox, ApoB100, ApoA1	
3	4 mL LH (green)	Insulin	
4	4 mL FX (grey)	Glucose	
5	4.5 mL citrate (light blue)	Isoprostanes	Pre-chilled, immediately transferred on ice
6	4.5 mL citrate (light blue)	Isoprostanes	
7	4.5 mL EDTA (lavender)	EPC*, Erythrocyte lipids	Tourniquet released
8	4.5 mL EDTA (lavender)	Prostacyclin	
9	4.5 mL citrate (light blue)	PMA*	

Table 2-2. Outline of blood sampling

CRP, C-reactive protein; FLIP, full lipid profile (TAG, NEFA, total cholesterol, LDL, HDL); NOx, nitrates/nitrites; EPC, endothelial progenitor cells; PMA, platelet monocyte aggregates.

* analysis on fresh blood.

Tubes 3, 4 and 8 were centrifuged at 1500 g for 15 min at 4 ºC less than 10 min after blood sampling. Tubes 1 and 2 were allowed to stand at room temperature for 30 min before being centrifuged in the same conditions. Blood for 8-iso-$PGF_{2\alpha}$ analysis was drawn into chilled citrated tubes (tubes 5 and 6) and chilled fresh indomethacin was immediately added (final concentration 15 µmol/L). The sample was kept on ice 30 min prior to centrifugation at 2400 × g; 15min. BHT was added (final concentrations 20 µmol/L) and the samples stored at -80°C until analysis (detailed protocol in Appendix XXVII). Tubes 7 and 9 were used for fresh whole blood analysis by flow cytometry. Because cold temperature may activate platelets, PMA tubes were kept at room temperature to be analysed less than 20 min after blood sampling according to the protocol described in 2.2.13.3. EPC tubes were kept on ice and analysed within 30 min as described in 2.2.13.2. The 2 experiments on fresh blood were run in parallel and samples were read on a flow cytometer. Once whole blood was taken for PMA and EPC treatment, the EDTA and citrated tubes were centrifuged at 1500 g for 15 min at 4 ºC for the purpose of providing

spare plasma samples. Plasma and serum samples from the 9 tubes were aliquoted and frozen at either -40ºC or -80ºC (see Appendix XXVIII). The red cells obtained from centrifugation of the EPC tube (tube 7, EDTA) were kept in the fridge 3 to 5 days until they were treated for erythrocyte lipid analysis as described in 2.2.6.

At the end of the 2 h visit, the participants were provided with a light meal to consume before leaving the metabolic unit.

2.2.6 Erythrocyte lipids

Erythrocyte lipids were measured in order to determine the omega-3 index (erythrocyte EPA+DHA) as a marker of compliance, in addition to the capsules count. Blood was collected in 4.5 ml EDTA tubes and centrifuged at 1300 g, 4ºC for 15 min as described earlier. The pellet (erythrocytes) was kept in the fridge 3 to 5 days before being treated according to the following protocol, adapted from Rose and Oklander, 1965 (Rose and Oklander, 1965).

2.2.6.1 Lipid extraction

Erythrocytes lipids: The buffy coat was removed, and the erythrocytes washed three times with 5 volumes of cold saline (0.89%) for 10 min at 900 g, 4ºC. 0.5 ml erythrocytes were pipetted into large 25 ml glass-stoppered centrifuge tubes and 0.5 ml distilled water added. The contents were vortex-mixed to haemolyse the erythrocytes. 5.5 ml chilled isopropanol were then added slowly, and the tube was vortexed thoroughly again. After 15 min storage at 4ºC, 3.5 ml chilled chloroform were added and the contents mixed thoroughly. At the end of another 30 min at 4ºC, the samples were centrifuged at 1500 g for 15 min at 4ºC. The supernatant was collected and stored in glass tubes at -40ºC until GC analysis.

2.2.6.2 Methyl ester preparation

Lipid extracts were treated in 3 batches; lipid extracts from the same subjects were treated at the same time.

Approximately 1 ml of each thawed lipid extract was transferred to a labelled GC vial and evaporated in an eppendorff concentrator at 45 ºC for ~1 h. Each extract was then re-dissolved in 100 µl hexane, and 50 µl $NaOCH_3$ added to neutralise acidity. The content was thoroughly mixed and 1 ml hexane was added. $CaCl_2$ was then added in order to

absorb any remaining water and the vials were covered and allowed to stand 1 h at RT. Samples were transferred to new GC vials, and evaporated for 20 min at RT. The final extracts were re-dissolved in 50 µl hexane and transferred to inserts in GC microvials for GC analysis.

2.2.6.3 Gas chromatography (GC) conditions

Fatty acid methyl esters were separated on an Agilent 6890 Gas Chromatograph (Agilent Technologies) fitted with a flame ionization detector with a 25 m BP75 capillary column. The injection volume was 2 µL, the temperature was 160ºC for 4 min and then rose to 200ºC in 10 min (gradient of 12ºC/min).

2.2.7 Non esterified FA (NEFA) profiles

NEFA patterns were measured in order to relate to the FA profiles used in the cell cultures work described in Chapter 4, representing NEFA profiles. Compared to erythrocyte, which give an indication of longer term FA intake over the last month, NEFA profiles give an index of compliance in the short term, i.e. the last few days of intervention. Blood was collected in 4.5 ml EDTA tubes and centrifuged at 1300 g, 4ºC for 15 min (see 2.2.7). Plasma was aliquotted and stored at -80ºC until analysis. The NEFA were extracted and isolated as previously described (Burdge, et al., 2000, Folch, et al., 1957, Lepage and Roy, 1988, Madsen, et al., 2011).

2.2.7.1 Lipid extraction

0.5 mL of plasma was mixed with 0.5 mL internal standard (C15:0) and 10 mL of $CHCl_3$:MeOH (2:1) + BHT 50mg/mL were added. The content was gently mixed for 15 minutes on a roller mixer and 1.5 mL of 0.89% saline was added. Tubes were then vortexed and centrifuged at 1,600 g for 10 minutes at 4°C. The upper aqueous phase was discarded and the lower organic phase collected. The protein disk was reextracted following the same procedure. The two organic phases were combined and dried under nitrogen.

2.2.7.2 Thin layer chromatography (TLC)

The dry extract was redissolved in 50 µL $CHCl_3$ and applied on TLC plate. Oleic acid was used as an external standard. The plate was placed in a tank and developing solution (hexane : diethylether : acetic acid, 80:20:1, v/v/v) was added. The tank was closed and the plate allowed to develop for approximatively 50min. Cholesterol esters migrate to

the solvent front, followed by TAG, NEFA, cholesterol, diacylglycerols, monoglycerols and phospholipids. Once the solvent reached the top of the plate, the plate was dried under nitrogen and sprayed with 2-7-dichlorofluorescein in 95% methanol for visualisation under UV light. The NEFA band was identified by comparison with the external standard band and scraped into a screw cap glass tube containing the methylating solution.

2.2.7.3 Methyl ester preparation

The extract was dissolved in 2mL toluene : methanol : acetyl chloride, 20:80:10 (v/v) (methylating agent). Tubes were sealed and incubated overnight at 40 ºC in water bath. 5 mL of 6 % K2CO3 solution were added and themixtures centrifuged at centrifuged at 1,600 g for 10 minutes at 4°C. The upper phase was evaporated in the eppendorff concentrator, redissolved in 40µL hexane and transferred to insert for GC analysis.

2.2.7.4 GC conditions

Fatty acid methyl esters were separated on an Agilent 6890 Gas Chromatograph fitted with a flame ionization detector with a 25 m BP75 capillary column. The injection volume was 2 µL, the temperature was 160ºC for 4 min and then rose to 200ºC in 10 min (gradient of 12ºC/min).

2.2.8 Lipid profiles and glycaemic control

Blood was collected into tubes containing clot activator and gel for lipid analysis, FX and LH tubes for glucose and insulin analysis, respectively, as described in 2.2.5. Plasma and serum were frozen at -80ºC on the day of visit until analysis at KCH under the supervision of Dr Roy Sherwood. TAG, cholesterol, and HDL concentrations were measured as described in 2.2.4 and LDL cholesterol was calculated using the Friedewald formula. NEFA were measured using an enzymatic colorimetric method assay (NEFA-HR(2) Assay kit, WAKO Chemicals Gmbh, Fuggerstrabe 12, D-41468 Neuss, Germany) as described in Appendix XXIX. Briefly NEFA is converted into Acyl-CoA by Acyl-CoA synthetase (ACS), which is then oxidised by Acyl-CoA oxidase (ACOD) to form 2,3-trans-Enoyl-CoA and hydrogen peroxide. The latter yields a blue purple pigment in the presence of peroxidase (POD), which is proportional to the initial NEFA concentration. Glucose was determined by the hexokinase and G6PD method as described in 2.2.4. Insulin was measured by ELISA on the ADVIA Centaur® XP immune assay system (Siemens

Healthcare Diagnostics Ltd, Surrey, UK), as described in Appendix XXX. Insulin resistance was assessed by the homeostatic model assessment (HOMA-IR: Glucose mM x Insulin mU/L / 22.5) and the quantitative insulin sensitivity check index (QUICKI: 1 / (log(fasting insulin μU/mL) + log(fasting glucose mg/dL)).

2.2.9 Nitrate and Nitrite measurements

In vivo and *in vitro*, NO interacts with biological fluids to form both nitrite (NO_2^-) and nitrate (NO_3^-), making necessary to convert nitrate to nitrite to measure total NO. Thus, total NO (NOx = nitrate + nitrite) is measured in a two-step reaction:

1- Nitrate is converted to nitrite by the nitrate reductase and its cofactor NADPH,H^+. The resulting nitrite measured corresponds to the total NO present in the medium.
2- Nitrite is complexed to a reagent for detection. Two main techniques are available for this step:
- complexation with the Griess reagents (sulfanilamine and then N-(1-Naphtyl) ethylenediamine) that convert nitrite to a deep purple azo compound.
- complexation with a fluorometric reagent (diaminonaphtalene: DAN) followed by an alkalinisation that enhances the detection.

Here total NO was measured by a nitric oxide ELISA kit using Griess reagents for detection, following the manufacturer's instructions (R&D System). Plasma sample was first passed through a centrifugal filter device (NMWL: 10kDa) to remove proteins which may affect the reading of the signal in the NO assay. It was then diluted 2 times in assay buffer prior to the experiment. 50 μL of each plasma sample or nitrate standards (diluted in assay buffer) were transferred to a 96 well plate in duplicate, to which 50 μL of assay buffer were added. Then 25 μL of cofactors (NADPH, H^+) and 25 μL of nitrate reductase were added. The plate was covered, shaken and incubated 30min at 37ºC. Then 50 μL of each Griess reagent (I and II) were added. The plate was covered, shaken again and allowed to develop for 10min at RT before the absorbance was read at λ = 540 nm.

2.2.10 Isoprostanes

8-iso-prostaglandin-F2α (8-IsoP-F2α), a prostaglandin F2-like compound biosynthesized nonenzymatically by a free-radical oxygenation of arachidonic acid, was

measured in plasma in order to assess oxidative stress. The procedure for isoprostane analysis comprised the alkaline hydrolysis (saponification) of the plasma 8-isoprostanes, followed by a purification by immunoaffinity columns. The isoprostane salts were then subjected to esterification with PFBBr, followed by silylation with BSTFA (derivitisation). The resulting derivative was detected and quantified by GC-NCI-MS. The method of internal standardisation was used for quantification.

Samples were treated in 5 batches; samples from the same subject were treated in the same batch.

2.2.10.1 Alkaline Hydrolysis Protocol

25µ1 (1ng) of internal standard working solution was added to 1.5mL-2ml of EDTA plasma and gently mixed. 1 mL of 15% w/v KOH was added and the resulting mixture was mixed and incubated for 1 hour at 60°C on a heating block.

2.2.10.2 Purification Protocol

Prior to the purification procedure, affinity columns were connected to the Vacmaster processing station and the storage solution allowed to drain out and the heating block was set to 60˚C. In each step, washing solutions, buffer and sample solutions were passed through the affinity column through gravity alone.

The hydrolysate was allowed to cool down and neutralized with 3-4 mL of 1M KH_2PO_4. The volume of KH_2PO_4 was adjusted to reach a pH of 7-7.4. 2 mL of affinity column buffer solution were added and each mixture was gently decanted into each labeled column. 2ml of column buffer were added to the column in order to remove non specific binding, followed by 2ml of ultrapure water to wash out remaining salts. Washings were discarded and the 8-isoprostane were eluted by adding 2ml elution solution. The collected 2 mL sample was evaporated to dryness in a vacuum centrifugal concentrator. In the mean time affinity columns were regenerated by washing with 5 mL UltraPure water followed by 5 mL column buffer. Columns were stored in 1-2 mL column buffer solution in the upright position at 4°C to prevent the packing material from becoming dry. Such columns may be used up to five times.

2.2.10.3 Derivitisation Protocol

All operations were conducted in a fume cupboard. 25µl of 10%v/v DIPEA, followed by 25µl of 20%v/v PFBBr were added to the dry residue and the mixture was gently mixed and incubated in the heating block for 10min at 60°C. The samples were allowed to cool down and evaporated to dryness under a nitrogen stream at room temperature. 50µl of BSTFA were then added, followed by 5µl of 10%v/v DIPEA and the mixture was gently mixed and incubated for 4 min at 60°C. The samples were allowed to cool down and evaporated to dryness under nitrogen. The residue was dissolved in 20µl of isooctane and transferred to GC insert vials for GC-MS analysis.

Preparation of Calibration standards

5 calibration standards were prepared. 10, 20, 200, and 400 µL of iso-8-$PGF_{2\alpha}$ (external standard, 10µg/mL) were mixed with 20 µL (1ng) of iso-8-$PGF_{2\alpha}D_4$ (internal standard, 1µg/mL), evaporated to dryness and derivitised as described above.

2.2.10.4 GC- Mass spectroscopy (MS) analysis

The operating parameters and detailed procedure is fully described in Appendix XXXI. Helium was used as a carrier gas and negative chemical ionization (NCI) used methane as a reagent gas. The initial column temperature of 80°C was held for 1.8 min and then programmed from 80 to 235°C at 34°C/min, then increased to 280°C at 10.3°C/min and maintained at 280°C for 10 min, giving a total run time of ~21 min. The F2-isoprostanes were detected by selective ion monitoring (SIM) using m/z 569 and m/z 573 for iso-8-$PGF_{2\alpha}$ and iso-8-$PGF_{2\alpha}D_4$, respectively. Peak identification was based on comparison of retention times with standards.

2.2.11 Blood pressure (BP) and heart rate (HR)

At screening, BP and HR were measured after 5 min rest in the seated position with an automatic upper arm blood pressure monitor (Omron 705IT). The measurements were done in triplicate with 3 to 5 min intervals. An additional measurement was made when one value varied from more than 15% from another, and the average of the 3 closest values was taken. On study days, BP and HR were also assessed after 15 min rest in the supine position at each PWA measurement (triplicate). The DVP analysis, performed in alternating order

with the PWA measurement, provided an additional measure of supine HR in triplicate (see Figure 2-3 for the progress of measurements during the study day). For BP and HR measured in the supine position, the average of 3 measurements was taken, unless % COV of the 3 recordings was >15%, in which case the average of the 2 closest was taken. The measurements were made by Miss Aseel Alsaleh, the data entry and analysis were carried out by both Aseel and the author.

ABP was recorded 2 to 3 days before each visit day. The instrument was programmed to record SBP, DBP, MAP and HR every 30 min during the day (7 am-10 pm) and every hour at night (10 pm-7 am). Subjects were asked to engage in routine daily activities and write down what they were doing (walking, sitting, standing, exercising) at the time of each blood pressure cuff inflation, as well as the times that they were sleeping, on a diary card (Appendix XXIV). Data were transferred to a computer and analysed using the software provided by the manufacturer (A&D Instrument Ltd, Abingdon, U.K.). The mean of 24 h measurements, as well as mean day-time and night-time measurements were calculated based on the subjects' times of sleep from the diary card. Explanations on how to use the monitor and fill in the diary card were given by the author; data entry and analysis were carried out by both Miss Aseel Alsaleh and the author.

2.2.12 Vascular measurements

All the non-invasive vascular measurements (PWA, DVP, capillaroscopy) were made in the supine position and were carried out on the left arm, while BP was measured on the right arm (see Figure 2-3 for the progress of vascular measurements on the study day).

2.2.12.1 Radial Pulse Wave Analysis (PWA)

SphygmoCor Pulse Wave Analysis (PWA) uses the principles of applanation tonometry as used in ocular tonometry (Garber, 1999) to record non invasively the peripheral arterial waveform from the radial pulse. It applies a convolutional algorithm and a generalized transfer function (characteric of the hydraulic properties of the upper limb vasculature) in order to obtain a derived central aortic waveform (O'Rourke, et al., 2001) (see chapter 1). Using a handheld probe, the radial artery was flattened (applanated) against a rigid underlying structure (bone). According to the Imbert-Fick law, the force per unit

area applied to flatten the artery equals the internal pressure of the artery. Thus, the force registered by the high-fidelity tonometer (strain gauge transducer, AtCor/Milar tonometer SPT-301B) at the tip of the probe is the true intra-arterial pressure of the radial artery. Applanation is appropriate if pulse waves are consistent, beat to beat, with a great amplitude, and if the pulse wave obtained corresponds to the one expected in an artery (sharp upstroke, straight rise to the first systolic peak, a definite sharp incisura, and near exponential pressure decay in late diastole). Measures of pressure and time obtained from the synthesized aortic waveform are used to assess parameters of peripheral and central haemodynamics (O'Rourke, et al., 2001). The computerized report on analysis of radial and aortic pressure waves, as obtained with Sphygmocor Px, is shown in Figure 2-4.

The principal stiffness parameter assessed by PWA is the augmentation index, representing the augmentation of pressure in the systolic phase induced by the reflected wave, as described in Figure 2-5. The augmentation can be expressed in mmHg (augmentation pressure, AP or ΔP) or as a percentage called augmentation index (AIx). ΔP is the difference of pressure between the reflected wave P2 and the forward wave P1. AIx can be expressed as P1 relative to P2, or ΔP relative to pulse pressure (PP) (Mills, et al., 2008). PWA calculates AIx from the pulse wave directly measured at the radial artery (peripheral AIx) and from the derived aortic pulse wave (central AIx).

The shape of the pulse wave varies throughout the arterial tree depending on the stiffness of the large arteries (Figure 2-5). In the compliant and elastic arteries of healthy young individuals, the pulse wave velocity is reduced so that the reflected wave P2 occurs in diastole. Therefore P2 is inferior to P1 and the augmentation ΔP is negative. In contrast, with arterial stiffening, as observed in ageing, SBP is elevated and pulse wave velocity increases, i.e. the time to wave reflection is shortened. This means that the peak of the reflected wave occurs in mid systole rather than diastole, augmenting the already high SBP, while removing major support of DBP (Kaplan, 2000). As a result, ΔP and AIx, as well as PP, are augmented.

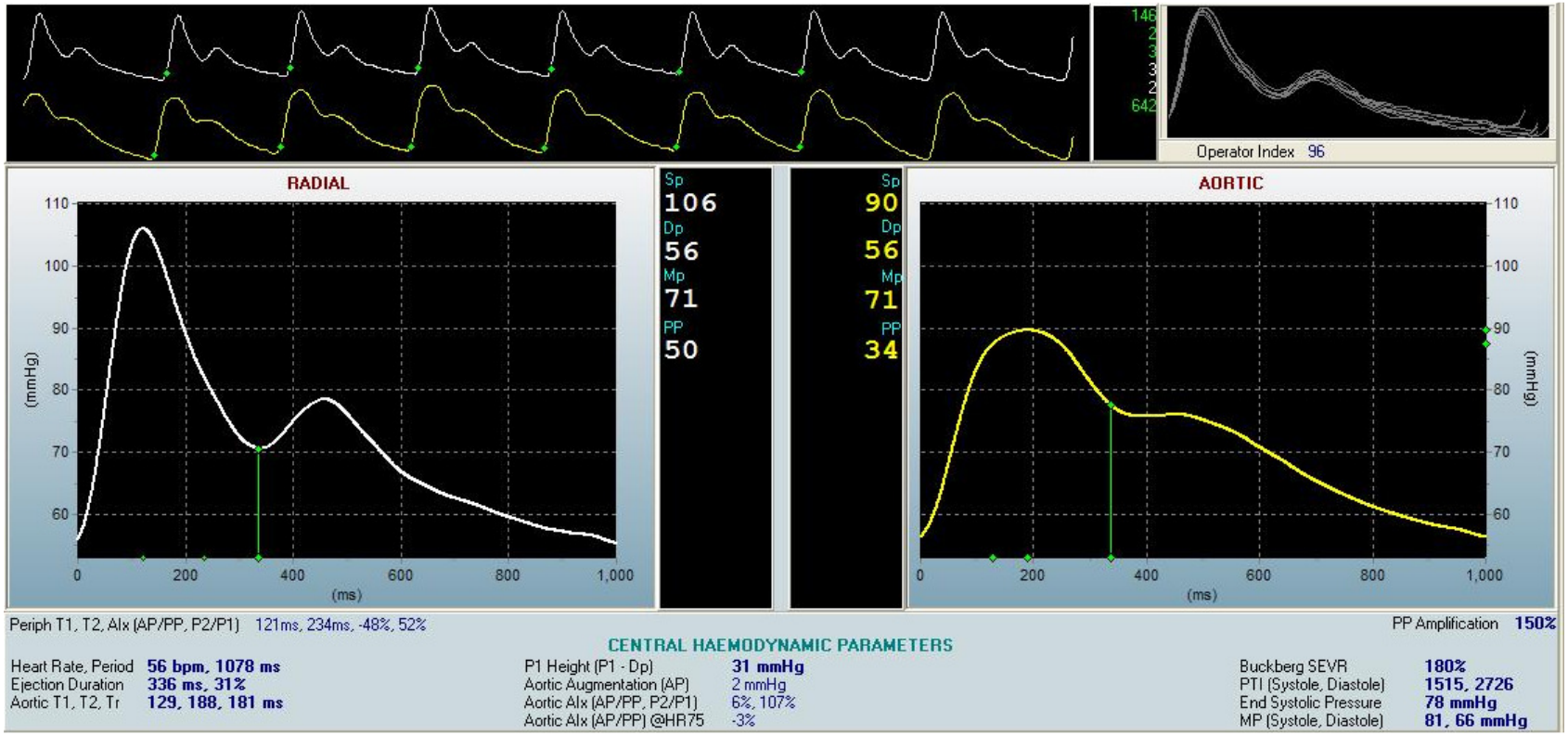

Figure 2-4 Radial artery and synthesized aortic pressure waves obtained by SphygmoCor Px (AtCor Medical)

A series of radial pressure waves, recorded over 8s (upper continuous trace) are used to synthesize a series of aortic pressure waves (lower continuous trace), using a generalised transfer function. Both radial and synthesized aortic waves are averaged into single waves (lower traces). Features of the waves including the foot, shoulder, peak, and incisura (shown in green) are identified automatically using differentials, flagged, and used to calculate peripheral and central haemodynamic parameters. The detailed report gives information relevant to ventricular/vascular interaction from both pressure and time values (lower part). The variability between the recorded wave forms and BP measurements is used to calculate the operator index as a quality control (upper right) (O'Rourke, et al., 2001).

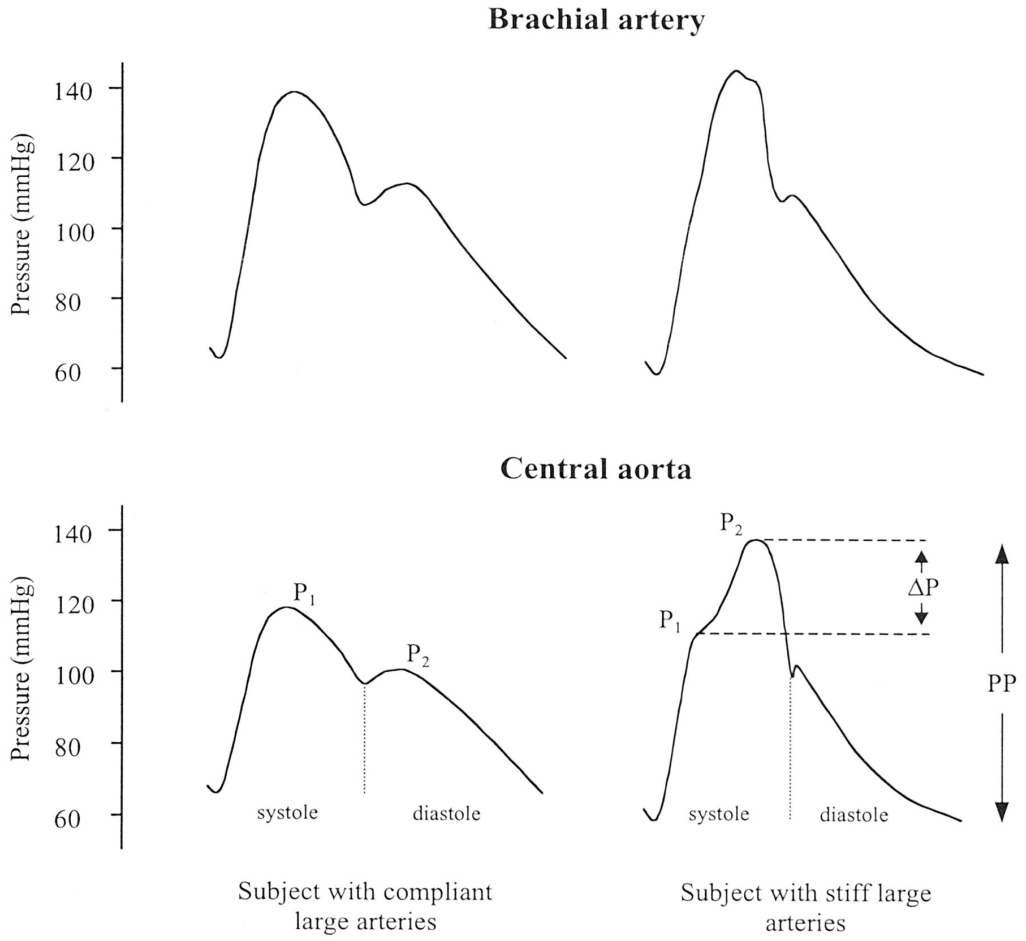

Figure 2-5 Schematic representation of pulse pressure amplification, as assessed by applanation tonometry (Oliver and Webb, 2003)

The augmentation pressure (ΔP) is the difference between the reflected pressure wave (P2) and the initial systolic pressure (P1) systolic peak (forward wave). The pulse pressure (PP) is the difference between systolic and diastolic blood pressure. The augmentation index (AIx) is given as the percentage of ΔP relative to PP, or of P2 relative to P1.

In compliant arteries (left), the reflected pressure waves occur in diastole and ΔP is negative (P2<P1). With the stiffening of the arteries (right), the reflected waves arrive earlier, augmenting SBP in the detriment of DBP, which leads to an increasingly positive ΔP (P2>P1).

2.2.12.1 Digital Volume Pulse (DVP) analysis

The DVP is an accurate and non invasive method used to obtain information on the pulse waveform in the finger, which allows the assessment of vascular tone and peripheral arterial stiffness (see chapter 1). DVP was recorded by measuring the transmission of infra-red light absorbed through the finger, which is directly proportional to the volume of blood in the finger pulp. The pulse waveform obtained in healthy individuals exhibits clearly defined two peaks (Figure 2-6). The systolic component results from the pressure directly transmitted from the aorta to the finger (direct wave). The diastolic component is formed by the pressure transmitted from the ventricle along the aorta to the lower body where it is reflected back along the aorta to the finger (reflected wave). The transit time between the two peaks (peak-to-peak time, PPT) is related to the stiffness of large arteries (stiffness index, SI). The amplitude of the reflected wave is mainly determined by the vascular tone in small arteries and provides the reflection index (RI) (Millasseau, et al., 2006). The DVP recorded and the indices calculated from the waveform are described in Figure 2-6. As observed with radial PWA, the shape of the wave form is altered with arterial stiffening: PPT is shortened, so that the second peak is attenuated and replaced by an inflection point in the down slope of the wave form. This inflection point is then used to calculate the (augmented) RI and SI (Millasseau, et al., 2006).

It should be noted that the shapes of the radial and digital pulses are similar (Millasseau, et al., 2000), and both SI (measured by DVP) and the peripheral AIx (measured by radial PWA) are representative of the stiffness of large peripheral arteries.

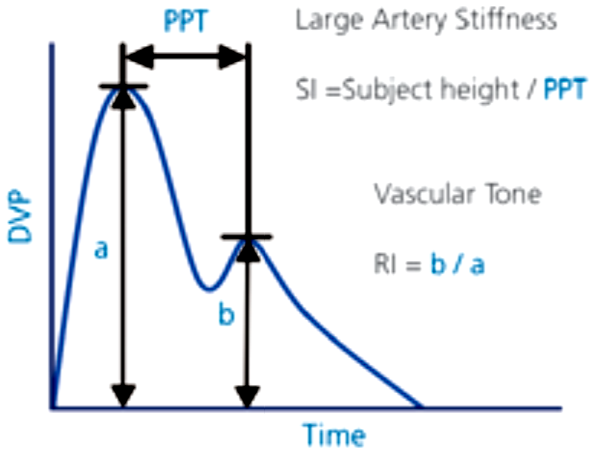

Figure 2-6: Determination of the stiffness index (SI) and the reflection index (RI) by contour analysis of the digital volume pulse (DVP)

SI is obtained by dividing the subject height (in m) by the peak-to-peak time (PPT, in s). It estimates the pulse wave velocity in large arteries and is a measure of large artery stiffness.

RI is expressed as the percentage of the height of the diastolic peak (a, reflected wave) relative to the systolic peak (b, direct wave). It is a measure of the systemic vascular tone (small arteries)

(Micro Medical Ltd, 2010)

2.2.12.2 Capillaroscopy

The CapiScope Image Acquisition and Analysis software (KK Research Technology) allows capturing and storing capillaroscopy images from video onto a Personal Computer. The laser beam can be positioned either far or next to the nail fold, giving images with different aspects and allowing different measurements (Figure 2-7). Capillaries at the nail fold are parallel to the skin and the blood flow can be visualised, allowing the calculation of red blood cell velocity (RBCV) using the spatial correlation technique. Capillaries further from the nail fold are perpendicular to the skin and appear like dots, which allows the calculation of capillary density, i.e. the number of capillaries per surface unit. In our study, we positioned the laser beam on 5 different spots chosen arbitrarily at the medium phalanx and far from the nail fold in order to calculate functional capillary density as previously described (Debbabi, et al., 2006).

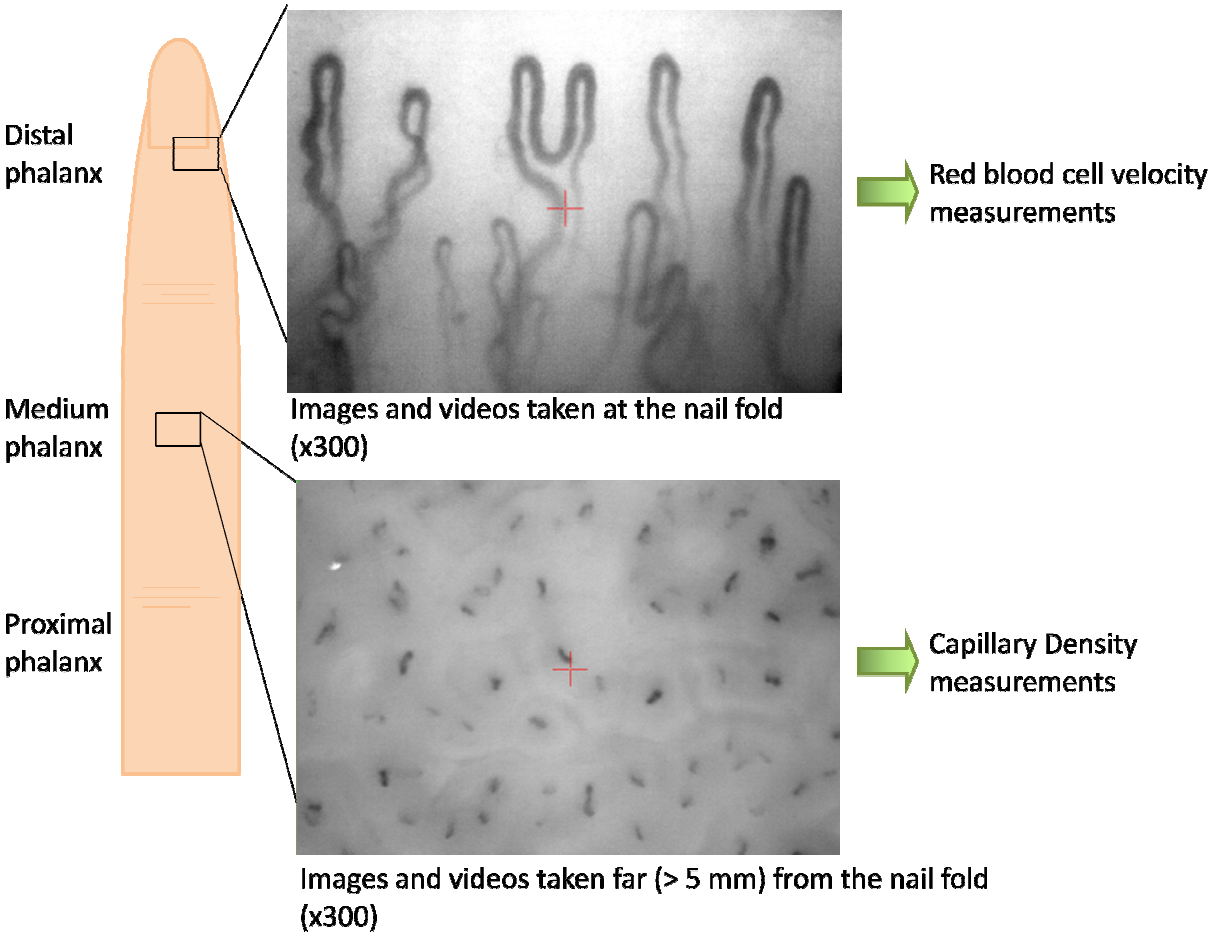

Figure 2-7: Images and videos obtained by CapiScope Capillaroscopy System (KK Research Technology Ltd, Devon, UK)

2.2.13 Flow cytometry analysis

2.2.13.1 Principle

Flow cytometry (Figure 2-8) uses the principles of light scattering, light excitation, and emission of fluorochrome molecules in order to detect particles or cells of diameters ranging from 0.5 μm to 40 μm. Fluorochrome-labelled cells from blood or other biological fluids are focused in a sheath of PBS so they intercept an optimally focused light source (usually laser) one by one. As they intercept the light source, cells scatter light and fluorochromes – either intra-cellular or at the surface of the cell - are excited to a higher energy state. Scattered light, as well as the light emitted by the excited fluorochromes, are then converted to an electrical signal by optical detectors. Light that is scattered in the forward direction (typically up to 20° offset from the laser beam) is collected by a lens known as the forward scatter (FS) channel, while the light that is deviated at a 90° angle is detected by the side scatter (SS) channel. Signal collected by FSC and SSC are proportional to the size and granulosity of the particle, respectively. The light released by the fluorochromes has spectral properties (emission wavelength) that are characteristic of the fluorescent dye used. The emitted light passes through specific optical filters, which block certain wavelengths while transmitting (passing) others, before being detected by the detectors, usually photomultiplier tubes (PMTs). The electrical pulses generated from scattered light (FS, SS) and fluorescence (FL-) by the detectors are then amplified, usually through linear and log amplifiers, respectively. The signals obtained are processed by an analog to digital converter (ADC), which allows the events (each one corresponding to one cell/particle) to be plotted on a graphical scale.

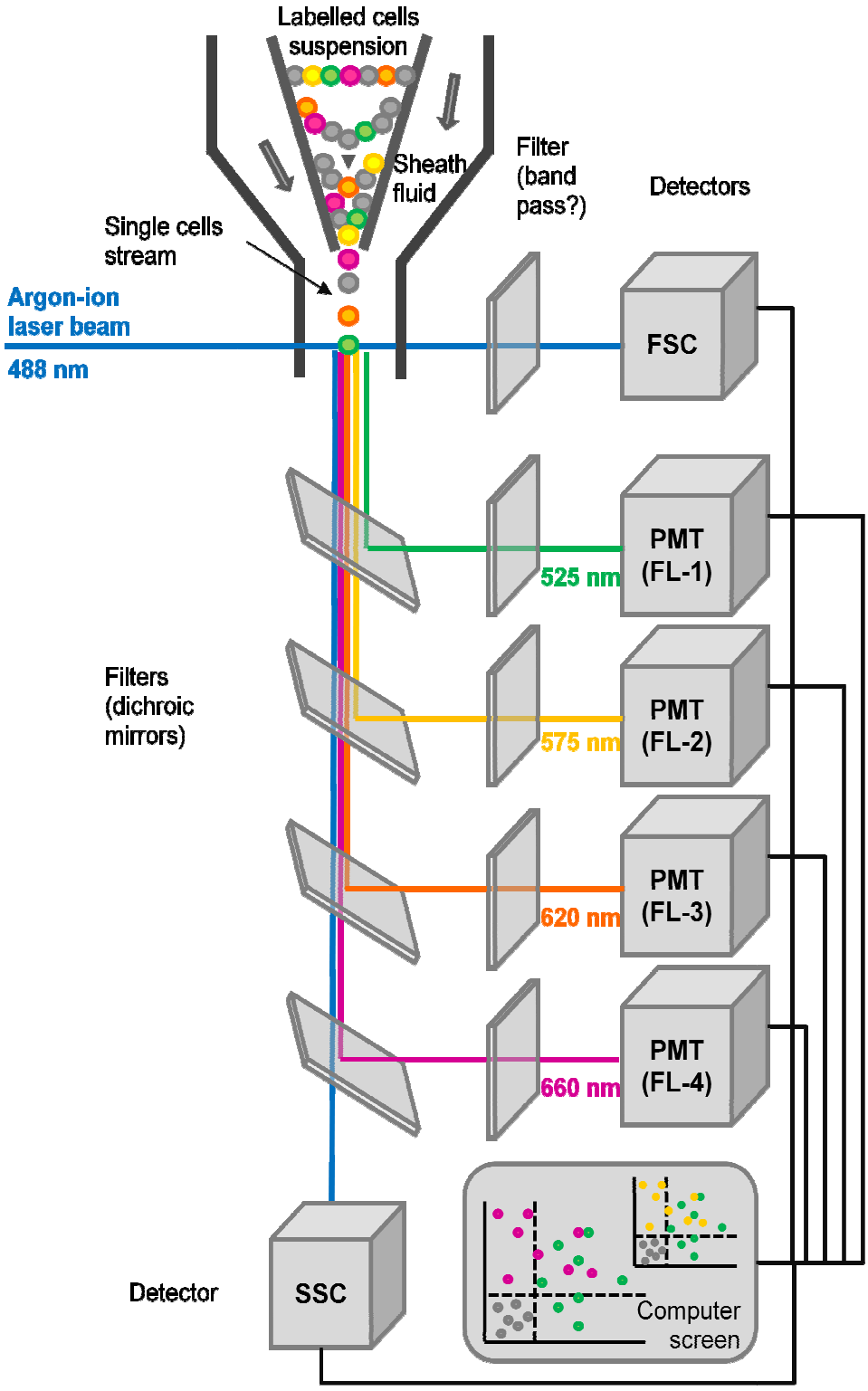

Figure 2-8 Schematic overview of a flow cytometer

FSC, forward scatter channel; SSC, side scatter channel; PMT, photomultiplier tube; FL, fluorescence channel.

Calibration of the flow cytometer before running the samples is composed of three steps:

1- An unstained blood sample is used to detect auto-fluorescence or background staining.
2- An isotype control is needed to detect non specific binding of the antibody (the antibody used is the same immunoglobulin isotype as the test antibody, but with a specificity that is irrelevant to the sample being analysed).
3- Colour compensation is required to correct the spectral overlap existing between two fluorochromes (the antibodies used are one testing antibody and the isotype control corresponding to the other testing antibody)

2.2.13.2 Endothelial Progenitor Cells (EPC)

As a transitory state between bone marrow cells and endothelial cells, EPC express different markers throughout their differentiation. CD133 and CD34 are early hematopoietic stem cell markers expressed on hematopoietic stem and progenitor cells from human bone marrow (Andrews, et al., 1989, Yin, et al., 1997). KDR is expressed all along EPC maturation and CD31 is characteristic of the mature endothelial phenotype, which EPC start expressing while becoming negative for CD133 (Hristov, et al., 2003). Various combinations of these markers (Figure 2-9) have been used to identify and count EPC (Nguyen, et al., 2012, Peichev, et al., 2000). In this experiment we used a method developed in our departments to identify two populations of EPCs, described as ‘early EPC’, which are KDR+/CD34+/CD133+, and ‘late EPCs’ that are KDR+/CD34+/CD31+. All two-markers combinations are reported, apart from CD31+/KDR+ cells, as it was considered that this population may not possess the progenitor characteristic of EPC.

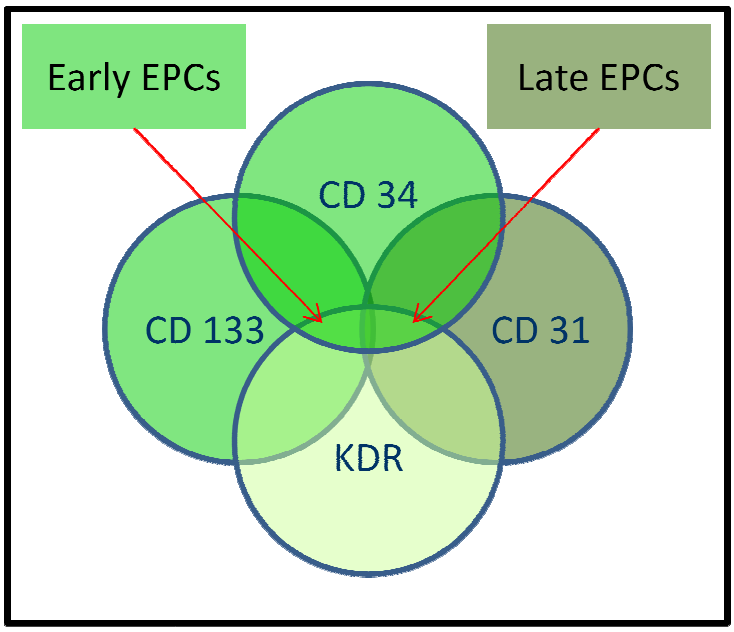

Figure 2-9 Identification of early and late endothelial progenitor cells (EPC)

EPC express KDR all along their maturation. Early EPC initially express bone marrow immature markers (CD133, CD34) but lose the expression of CD133 while they become positive for CD31, expressed by the mature EC, when can be defined as late EPC.

- *Sample preparation*

EDTA whole blood samples were kept on ice and analysed within 30 min of blood draw. From the first participant, six tubes were prepared containing the antibodies as listed below, volumes were defined following the manufacturer's instructions. When two participants had their visit on the same day, only tubes 5 and 6 were prepared from the second subject:

Tube 1, unstained sample: No antibody was added
Tube 2, isotype control: 20µl of IgG1 Fitc, 20µl of IgG1 PE, 10µl of IgG1 APC
Tube 3, colour compensation 1: 20µl of CD31 Fitc, 20µl of IgG1 PE
Tube 4, colour compensation 2: 20µl of CD45 PE, 20µl of IgG1 Fitc
Tube 5, sample: 20µl of CD31-Fitc, 20µl of CD34-PE, 10µl of KDR-APC
Tube 6, sample: 10µl of CD133- PE, 20µl of CD34- Fitc, 10µl of KDR-APC

100µl of whole blood were then transferred in each tube, the content was briefly vortex-mixed and incubated at room temperature for 20 min, sealed with parafilm and protected from the light. 2 mL of lysing buffer were added in each tube, the mixture was vortexed and incubated at RT until a clear solution appears (corresponding to the lysis of red cells). 25µl of fixative were then added, the content vortexed again and incubated for 10 min at RT. The tubes were then transferred to ice protected from the light until flow cytometry analysis (run on the same day). The acquisition time was 300s and the total events were 14227 +/- 6244 and 13516 +/- 5825 events for the first and second visit, respectively.

- *Gating Strategy*

The light transmitted in the forward direction is proportional to the volume of the cell, while the light scattered to the side is proportional to its granulosity. Each cell population in blood possesses a particular combination of size and granulosity, which can thus be identified according to their signal on a forward scatter (FS) vs. side scatter (SS) plot. An initial gate was set on mononuclear cells (gate A, Figure 2-10), which excludes the granulocyte population (high FS and SS). A second gate was set on a KDR vs. SS dot plot to include only the KDR+ events (gate N, Figure 2-10). Early and late EPCs were then identified on CD133 vs. CD34 and CD34 vs.CD31, in order to include the KDR+/CD34+/CD133+ and KDR+/CD34+/CD31+, respectively (P2 region, Figure 2-11).

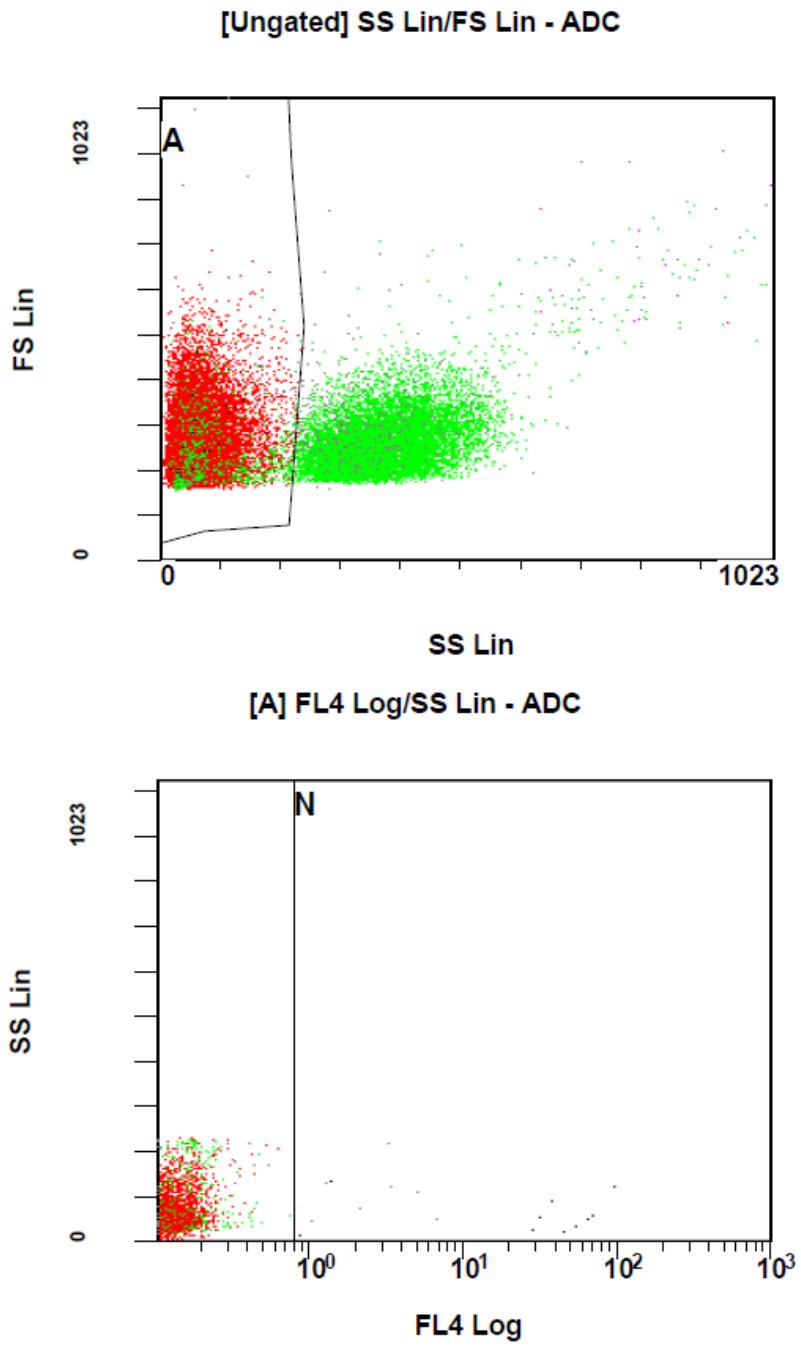

Figure 2-10 Identification of mononuclear cells and KDR+ cells

Mononuclear cells were gated according to their forward scatter (FS) and side scatter (SS) signal (Gate A). KDR+ cells were selected according to their fluorescence signal on FL-4 (Gate N)

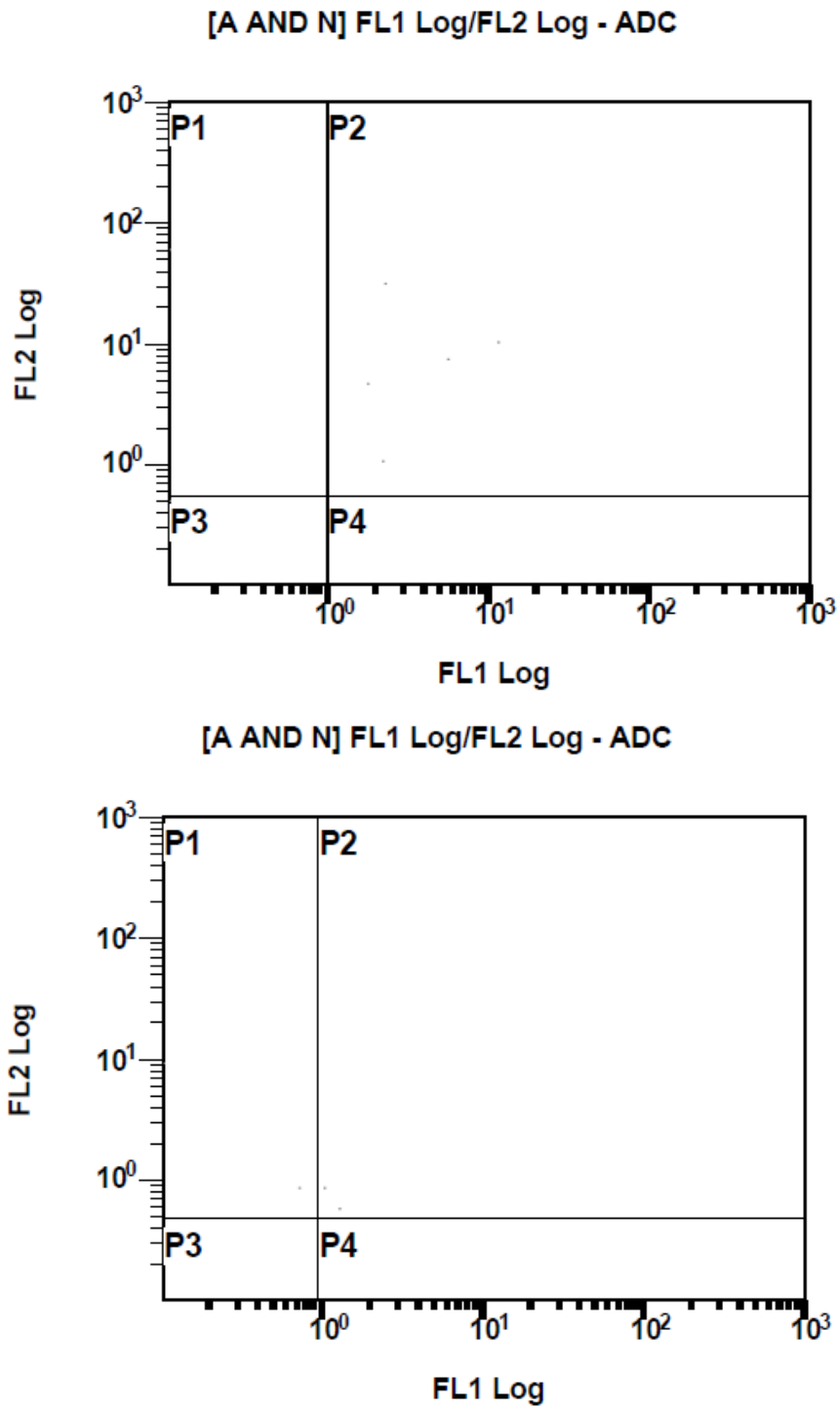

Figure 2-11 Identifications of 'early' (KDR+/CD34+/CD133+) and 'late' EPCs (KDR+/CD34+/CD31+)

EPCs are identified according to their fluorescence signal on FL1 and FL2 (P2 region), after gating on mononuclear cells (A) and KDR+ cells (N)

Early EPCs (left) are KDR+ cells that co-express CD34 (FL-1) and CD133 (FL-2)

Late EPCs (right) are KDR+ cells that co-express CD34 (FL-2) and CD31 (FL-1)

2.2.13.3 Platelet Monocyte Aggregates (PMA)

The endothelium plays a vital role in the regulation of blood flow, thrombosis and inflammation. Endothelium-derived anti-adhesive and anti-aggregant substances, including PGI_2 and NO, are known to inhibit platelet activation (Deanfield, et al., 2007). ED or vessel wall injury lead to the activation of platelets, of which platelet-monocyte-aggregates (PMA) are a sensitive marker (Michelson, et al., 2001), and were shown to inversely correlate with markers of EF in patients with stable CHD (Robinson, et al., 2006). The measurement of PMA by flow cytometry is a method which reduces *ex vivo* platelet activation to its minimum and is believed to represent platelet activation *in vivo* (Michelson, et al., 2001). The test was adapted from Goodall et al (Goodall and Appleby, 2004, Li, et al., 1999). PMA were identified by the co-expression of CD14 and CD42b (or P-selectin), specifically expressed by monocytes and activated platelets, respectively.

- *Sample preparation*

The study of PMA requires extra care in order to avoid *ex vivo* platelet activation and aggregation. Blood samples were treated within 15 min of draw at RT, and it was ensured that all reagents were at RT as well prior to the experiment. A blocking mouse immunoglobulin (MOPC31) was used to block Fc binding and non specific binding of monoclonal antibodies to leukocytes. In addition, a CD62P Monoclonal antibody blocker was used to block P-selectin binding which may encourage *ex-vivo* binding of platelets to leukocytes.

Appropriate volumes of HBS and antibodies and then whole blood were transferred to each tube as described in Table 2-3: Volumes of antibodies, buffer and blood required for PMA analysis by flow cytometry.

Tube	HBS Buffer	MOPC31 IgG1κ	Mab 9E1	IgG1-FITC	CD14-FITC	IgG1-PE	CD42b-PE	Blood (last)	Description
1	50 µl	-	-	-	-	-	-	50 µl	Blood only
2	50 µl	5 µl	2 µl	5 µl	-	-	-	50 µl	Isotype Control
3	50 µl	5 µl	2 µl	-	-	5 µl	-	50 µl	Isotype Control
4	50 µl	5 µl	2 µl	5 µl	-	-	5 µl	50 µl	Colour compensation
5	50 µl	5 µl	2 µl	-	5 µl	5 µl	-	50 µl	Colour compensation
6	50 µl	5 µl	2 µl	-	5 µl	-	5 µl	50 µl	Sample
7	50 µl	5 µl	2 µl	-	5 µl	-	5 µl	50 µl	Sample

Table 2-3: Volumes of antibodies, buffer and blood required for PMA analysis by flow cytometry

HBS, Hepes buffer saline; MOPC31, IgG1, Kappa from murine myeloma (clone MOPC31); MAb 9E1, CD62P monoclonal antibody blocker (clone 9E1)

The 7 mixtures were incubated 30 min at RT, and 250 µl of Optilyse®C solution were added. Tubes were allowed to stand at RT for 15 min and 250 µl PBS were added to stop the red cell lysis. Samples were allowed to stand another 5 min at RT prior to the flow cytometry analysis. The acquisition time was 200s and the total events were 25874 +/- 9180 and 24968 +/- 9024 events for the first and second visit, respectively.

- *Gating Strategy*

The monocyte population was identified by their specific expression of CD14, identified on the FL4 fluorescence channel. PMA were determined as events co-expressing CD62b – specific for activated platelets, on FL2 – and CD14. The PMA (CD62b+/CD14+) were measured as percentage of total CD14+ (Goodall and Appleby, 2004). The FS/SS and two-colour analysis dot plots are shown in Figure 2-12.

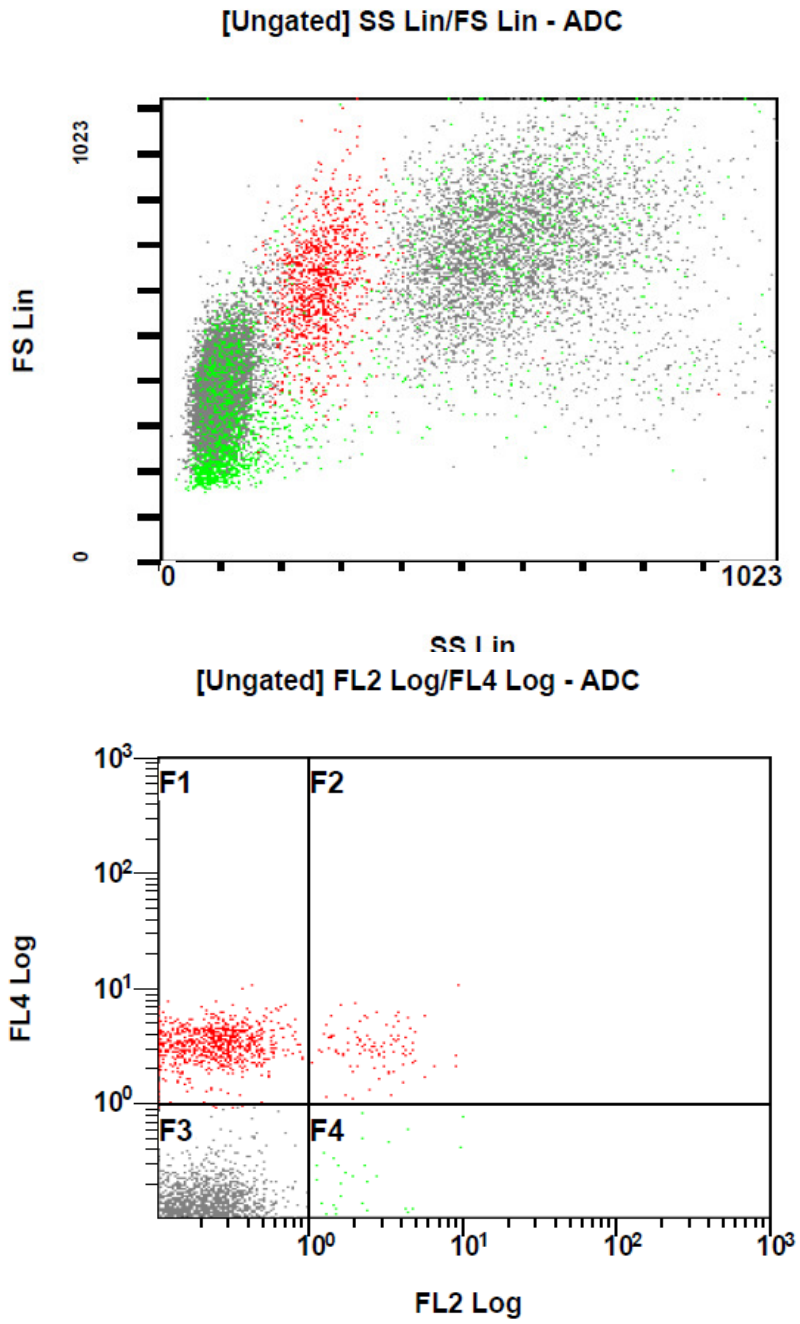

Figure 2-12 Identification of platelet monocyte aggregates (PMA) by flow cytometry

Monocytes (CD14+) and platelets (CD42b) show a positive fluorescence signal on FL4 and FL2, and appear in red and green, respectively.

Left: Total events from peripheral blood are plotted on a forward scatter (FS) vs. side scatter (SS) dot plot.

Right: PMA co-express CD14+ (FL4) and CD42b (FL2) and appear in the F2 region. Total events found in F1+F2 are monocytes while total platelets are identified in F2+F4.

2.2.1 Statistical analysis

The capillary density and endothelial progenitor cells measurements, novel markers of endothelial function, were defined as primary outcomes. However, these variables have rarely been investigated in relation to dietary interventions when the present study was in the planning stages, and the author was involved in setting these methodologies up in the Diabetes and Nutritional Sciences Division at KCL so prior data was unavailable. It was decided to base the power calculation on a related outcome variable, platelet monocyte aggregates (PMA). ED is associated with platelet activation (Deanfield, et al., 2007, Gkaliagkousi, et al., 2009), systolic blood pressure is closely related to PMA in blood, and an increase in PMA would indicate an impairment in nitric oxide signalling (Gkaliagkousi, et al., 2009). Standard deviations (10%) and expected change was taken from a previous paper (Din, et al., 2008) showing that a 4-wk oily fish intervention (500 g fish/wk, equivalent to 1g EPA+DHA/day) decreased PMA by 35%. It was calculated that a sample size of 16 in each group has an 80% power to detect a difference between means of 10% PMA, and a 99% power to detect 16% changes with a significance level (alpha) of 0.05. Data were analysed by analysis of covariance (ANCOVA) of endpoint values, adjusted for ethnicity, age, BMI and baseline value. Normality of the residuals was checked for analysis and log transformation of the data was attempted when required. If the residuals of the log-transformed data were still not normally distributed, non-parametrical analysis was performed (Kruskal-Wallis). Results were expressed as means (SD) and changes from baseline as differences between the means with 95% confidence intervals. When data were log transformed, the results were expressed as geometric means (SD) and the changes as ratios of the geometric means with 95% confidence intervals. In addition, paired t-tests were performed within each group and data were log-transformed when they did not follow a normal distribution as measured by the d'Agostino and Pearson coefficient. If data were still not normally distributed, a Wilcoxon matched-pairs test was performed. All statistical analysis was carried out with SPSS for Windows (version 17.0, SPSS, Chicago, IL) on the intention-to-treat basis. When a significant difference was observed between or within groups, bivariate correlations were performed for the values at 6 weeks and for the changes from baseline, in order to see if this related to changes in the main VLC *n*-3 PUFA (together or individually: EPA, DHA, DPA, EPA+DHA, EPA+DHA+DPA) in erythrocytes and NEFA. The significant correlations obtained were re-analysed by partial correlation analysis controlling for age, BMI and

ethnicity. The partial coefficient *r* and the *P* value are only given for the correlations that remained significant after controlling for the confounding factors. GraphPad Prism version 5.0 was used for designing graphs and measuring the d'Agostino and Pearson coefficient. Outliers were identified using the box-plot method (version 17.0, SPSS, Chicago, IL).

Chapter 3 The EPA and DHA trial: The differential effect of EPA and DHA on vascular and endothelial function

3.1 Introduction

Amongst the different types of fatty acids, *n*-3 PUFA, especially from fish oils, appear to be the most potent in improving cardiovascular health. Some studies suggested that ALA has cardioprotective effects, but evidence is not as robust as for EPA and DHA and there is insufficient data to encourage increasing ALA consumption (Wendland, et al., 2006) in order to reduce CVD risk. There is compelling evidence from epidemiological studies that fish oils, mainly composed of EPA and DHA, reduce the risk of CVD such as fatal coronary heart disease (CHD) (He, et al., 2004, Hu and Willett, 2002) and stroke (He, et al., 2004, Keli, et al., 1994). Several meta analyses have concluded that fish oils exert hypotensive, hypotriglycerideamic and anti-thrombotic actions (Balk, et al., 2006, Eslick, et al., 2009, Geleijnse, et al., 2002, Hartweg, et al., 2007, Morris, et al., 1993). Recent evidence also suggest that fish oil consumption improves arterial stiffness (Pase, et al., 2011) and endothelial function in some studies (Chin, et al., 1993, De Berrazueta, et al., 2009, Goodfellow, et al., 2000, Khan, et al., 2003, Morgan, et al., 2006, Schiano, et al., 2008, Shah, et al., 2007, Walser, et al., 2006, Wright, et al., 2008), although it remains uncertain in healthy subjects (Hjelte, et al., 2005, Sanders, et al., 2011, Theobald, et al., 2007, Ueshima, et al., 2007). While there is compelling evidence from animal and *in vitro* studies, as well as epidemiological studies, that fish oils exert anti-inflammatory (Farzaneh-Far, et al., 2009, Ferrucci, et al., 2006) and insulin sensitising (Arai, et al., 2009, Lombardo, et al., 2007, Nigam, et al., 2009, Thorseng, et al., 2009, Yanagisawa, et al., 2010) actions, randomised controlled trials in humans have given mixed results (Balk, et al., 2006, Hartweg, et al., 2008). The inconsistency may be due to differences in study design, populations, doses or duration of studies. In addition, proportions of EPA and DHA may greatly vary from one supplement to another and contribute to the variability of the results. Although there has been a growing interest in the past decade to elucidate the differential effect of EPA and DHA on diverse markers of CVD risk, research in this field is only at its beginning and the independent effects of EPA and DHA remain

poorly understood. The effects of EPA and DHA on markers of CVD risk were recently reviewed by the author of this thesis , with an emphasis on the comparison between the two individual FAs (Cottin, et al., 2011). While both EPA and DHA decrease triacylglycerol levels (Buckley, et al., 2004, Egert, et al., 2009, Grimsgaard, et al., 1997, Mori, et al., 2000, Nestel, et al., 2002, Olano-Martin, et al., 2010, Park and Harris, 2003, Woodman, et al., 2002), DHA appears to be responsible for an increase in HDL and LDL particle size (Kelley, et al., 2007, Liao, et al., 2010, Mori, et al., 2000, Rambjor, et al., 1996), although this effect has sometimes been observed with EPA (Nozaki, et al., 1992). Evidence to date suggests that DHA is more efficient in decreasing blood pressure and heart rate compared to EPA (Grimsgaard, et al., 1998, Mori, et al., 1999, Stark and Holub, 2004, Theobald, et al., 2007). It is not yet clear whether differences exist between EPA and DHA in their effects on endothelial function (Engler, et al., 2004, Mori, et al., 2000, Okumura, et al., 2002, Theobald, et al., 2007), and EPA may be more potent in improving arterial stiffness (Satoh, et al., 2009, Theobald, et al., 2007, Tomiyama, et al., 2005), although this is not supported by all studies (Nestel, et al., 2002). The effects of EPA and/or DHA on diverse markers of CVD risk have been extensively reviewed (Anderson and Ma, 2009, Balligand, et al., 1995, Hall, 2009, Lawes, et al., 2003, Ueshima, et al., 2007) and the strength of evidence is summarised in Table 3-1, classified following previous recommendations from the WHO (Byers, et al., 2002, Tang, et al., 2008).

Novel biomarkers and techniques have emerged in the past decade to assess endothelial and vascular function, which may further the understanding of the effect of EPA and/or DHA on CV health. Endothelial progenitor cells (EPC) were identified in the late 90's as a population of bone marrow derived cells able to differentiate *in vitro* into endothelial cells (Asahara, et al., 1997). EPC present the ability to repair the injured endothelium (Clark, et al., 2012) and create capillary networks in areas of ischemia (Campbell, et al., 2012). They have been associated to CV risk and proposed as a potential diagnostic and prognostic tool (O'Dunn-Orto, et al., 2012, Van den Eynde, et al., 2012). To the authors' knowledge, only one study has been published on the effect of fish oils on EPC count, repoting that a 12-week supplementation with 4 g/d fish oils (2.7 g/d EPA+DHA) had no effect on the number of CD133+/KDR+ cells in type 2 diabetics (Wong, et al., 2010). Blood capillaries consist of endothelial cells and capillary density can be measured using capillary imaging technology (Capilloscope). It has been observed that capillary rarefaction occurs with the development of

hypertension, and that reduction of salt intake may improve capillary density (He, et al., 2010). However nutritional interventions remain limited in this field and there is, to our knowledge, no data regarding the effect of dietary FA on capillary density.

Table 3-1. Summary of strength of evidence for the effect of EPA and DHA on metabolic risk factors in humans

	Fish Oils (EPA+DHA)	EPA	DHA
Lipid metabolism			
TAG	C ↓	C ↓	C ↓
Total Cholesterol	P ↑	I	PS ↑
LDL	P ↑	I	PS ↑
HDL	P ↑	I	PS ↑
LDL and HDL size	P ↑	I	PS ↑
Haemodynamics			
Blood pressure	C ↓	I	P ↓
Heart rate	C ↓	I	P ↓
Heart rate variability	PS ↑	I	I
Vascular function			
Arterial stiffness	P ↓	PS ↓	I
Endothelial function	P ↑	PS ↑	I
Thrombosis/haemostasis			
Platelet activation	P ↓	PS ↓	PS ↓
Fibrinolytic activity	P =	I	P =
Fibrinogen	P =	I	P =
Factor VII	PS ↑	I	PS ↑
Glycaemic control			
Fasting glucose	P =	I	I
Fasting insulin	P =	I	I
HbA1c	P =	I	I
Insulin sensitivity	P =	I	I
Inflammation	I	I	I

HbA1c, Glycated haemoglobin; ↑, increase; ↓, decrease; =, no effect; C, convincing; P, probable; PS, possible; I, insufficient data.

References (**Anderson and Ma, 2009, Balligand, et al., 1995, Hall, 2009, Lawes, et al., 2003, Ueshima, et al., 2007**)

3.1.1 Aims and objectives

Relatively few studies have made a head to head comparison of DHA with EPA. The understanding of this differential effect may be of great interest in populations with low EPA intake such as vegetarians, who may choose to supplement their dietary intake of LC n-3 PUFA in the form of DHA-rich algal oil. This chapter aims to investigate the effect of supplementation with either EPA or DHA (3g/day, 6 weeks) in healthy young males on endothelial and vascular function, as well as other CVD risk factors such as lipid profiles (TAG, cholesterol, NEFA), glycaemic control (HOMA, QUICKI), oxidative stress and platelet aggregation and how this relates to changes in erythrocyte lipid and NEFA composition. The omega-3 index (erythrocyte EPA+DHA) was used as a marker of compliance. Endothelial progenitor cells - novel marker of endothelial function - were measured by flow cytometry, and capillary density was measured by capillaroscopy to assess changes in microvascular function.

3.1.2 Hypothesis

The primary outcome variables were a change in the proportion of EPCs and an increase in capillary density. We tested the hypothesis that DHA would improve endothelial and microvascular function (increase EPC and capillary density) to a greater extent than EPA. Secondary outcome variables included other measures of vascular function, platelet function, oxidative stress and glucose metabolism. It was also expected that DHA but not EPA would improve blood pressure, heart rate and NOx, and decrease isoprostanes and platelet-monocyte aggregates, and that both EPA and DHA would decrease TAG levels (O'Dunn-Orto, et al., 2012), while having little or no effect on lipoprotein levels. No significant changes in fasting glucose or an indirect marker of insulin resistance (HOMA-IR) were expected as we were investigating a young, healthy population.

3.1.3 Study design: Overview

The study design (Figure 3-1) and the methods have been described in Chapter 2. Briefly, each subject received 5g of test oil per day in gelatine coated capsules. The placebo treatment provided (18:1n-9+18:1n-7 3.8g, saturated fatty acids 0.7g, and linoleic acid 0.2g), the EPA treatment provide (3.0 EPA , 0.1g DPA, 0.7g DHA,0.1g SFA, 0.2gMUFA) and DHA (0.5g EPA, 0.2g DPA, 2.7g DHA, 0.2g SFA, 0.4g MUFA) (The full composition is described in Appendix VII). A sample size of 16 participants

per group gave 80% power to detect a 1 SD unit difference between groups. Statistical analysis was done on an intention to treat basis and comparison between treatment were done using an analysis of covariance as described in Chapter 2. Compliance was primarily assessed by the proportion of EPA+DHA in erythrocyte membrane lipids, and secondarily by counting returned capsules.

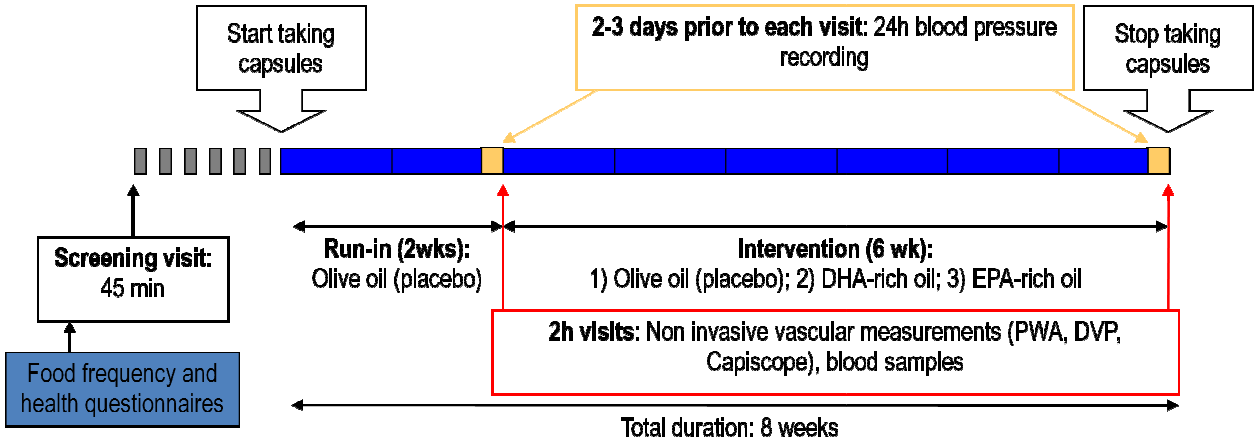

Figure 3-1: Outline of study protocol

3.2 Results

The CONSORT chart Figure 3-2 shows the flow of participants through the study, out of 57 participants, 49 were enrolled and randomized to treatment and 48 completed the 8-week intervention, data were available for analysis on 16 participants per group.

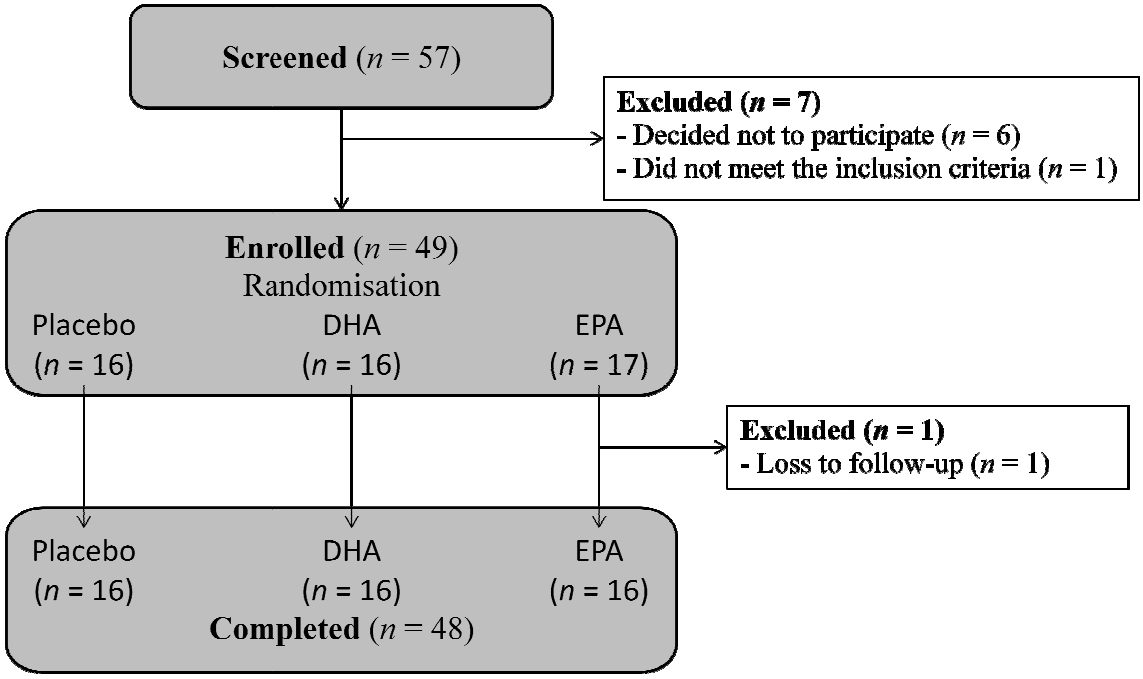

Figure 3-2: CONSORT flow chart of participants in the EPA and DHA trial

The details of the participants that completed the study are given in Table 3-2 and the randomized groups did not differ with the exception of plasma glucose concentration which was lower in the placebo group compare to the DHA group (P <0.05). Unused capsules were returned by 32/48 participants, 29 of whom returned fewer than 10% and were judged as 'good compliers' (Table 3-2).

Table 3-2: Characteristics of the participants at baseline and compliance to capsule intake

	Olive Oil (n = 16)	DHA (n = 16)	EPA (n = 16)	Total (n = 48)
Age, *y*	25.8 (7.4)	27.9 (8.0)	25.8 (7.5)	26.5 (7.5)
BMI, kg/m^2	23.6 (3.4)	23.3 (2.9)	22.9 (3.0)	23.3 (3.1)
Waist circumference, *cm*	81.7 (10.2)	82.5 (10.3)	78.4 (7.4)	80.9 (9.4)
Body fat, *%*	16.6 (5.5)	14.4 (4.5)	13.1 (4.4)	14.7 (4.9)
Systolic BP, *mmHg*	119.5 (10.4)	124.2 (10.7)	119.5 (13.5)	121.1 (11.5)
Diastolic BP, *mmHg*	66.7 (6.3)	70.9 (8.7)	67.9 (11.8)	68.5 (9.1)
HR, *bpm*	59.3 (6.2)	66.0 (10.4)	65.3 (8.5)	63.4 (8.9)
Height, *m*	1.76 (0.07)	1.75 (0.08)	1.79 (0.08)	1.77 (0.08)
Weight, *kg*	73.0 (12.2)	72.1 (12.5)	73.5 (11.2)	72.9 (11.7)
Plasma Glucose, *mmol/L*	4.9 (0.3)*	5.3 (0.4)*	5.1 (0.3)	5.1 (0.4)
Serum total cholesterol, *mmol/L*	4.6 (0.6)	4.4 (0.7)	4.5 (0.7)	4.5 (0.7)
Serum TAG†, *mmol/L*	0.8 (0.3)	0.8 (0.3)	0.9 (0.4)	0.8 (0.3)
Serum HDL-cholesterol†, *mmol/L*	1.4 (0.4) [1]	1.3 (0.31)	1.3 (0.2)	1.4 (0.3)
Serum LDL-cholesterol, *mmol/L*	2.7 (0.5)	2.7 (0.6)	2.7 (0.6)	2.7 (0.6)
Total cholesterol / HDL ratio	3.2 (0.7)	3.5 (0.8)	3.4 (0.8)	3.4 (0.8)
Subjects returning capsules	9	10	13	
Subjects returning <10% capsules	9/9	7/10	13/13	

Values are mean ± SD. BP, blood pressure; TAG, triacylglycerol

† geometric means; *$P < 0.05$ for significantly different values in the same row between the groups; one-way ANOVA test.

Body weight and body composition remained unchanged throughout the 6 weeks intervention (Table 3-3).

Table 3-3 : Metabolic parameters of the participants at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d).

	Olive Oil		DHA		EPA	
	Baseline	6 weeks	Baseline	6 weeks	Baseline	6 weeks
Weight, *kg*	72.03 (12.89)	73.04 (12.43)	71.56 (12.54)	73.47 (11.62)	74.21 (10.68)	73.74 (11.29)
BMI, kg/m^2	23.30 (3.75)	23.61 (3.51)	23.19 (2.99)	23.82 (2.66)	23.10 (2.80)	22.96 (3.01)
Fat composition, *% total mass*	16.63 (5.23)	16.46 (5.33)	14.73 (4.07)	14.22 (3.60)	13.92 (4.47)	13.72 (4.55)

Values are presented as mean (SD).

3.2.1 Erythrocyte lipids

Table 3-4 shows the composition of individual erythrocyte lipids at run-in and after six weeks on placebo, DHA or EPA treatment on the intention to treat basis. The proportions of linoleic acid (18:2n-6), dihomogammalinolenic acid (20:3n-6), arachidonic acid (20:4n-6), adrenic (22:4:n-6) and docosapentaenoic acid n-6 (22:5n-3) were lower and those of eicosapentaenoic acid (20:5n-3) and docosapentaenoic acid n-3 (22;5n-3) were greater following EPA vs placebo. The proportion of DHA was greater following DHA treatment compared with placebo. No other other significant differences vs placebo were noted. DHA differed compared with EPA for 20:3n-6, 20:4n-6, 22:4n—6 and 22:5n-6.

Table 3-4 Erythrocyte fatty acid composition at run-in and following placebo, DHA and EPA treatment (Intention to treat)

Fatty acid	Run-in n=47	Placebo=n=16	DHA=n=16	EPA=n=16	*P* value
16:0	17.0 (2.8)	17.5 (3.1)	17.0 (3.1)	18.4 (1.7)	0.226
16:1n-7	0.3 (0.1)	0.5 (0.5)	0.3 (0.1)	0.3 (0.1)	0.91
18:0	15.3 (1.5)	15.3 (1.1)	15.3 (1.9)	15.7 (1.3)	0.44
18:1 trans	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.675
18:1n-9	14.9 (1.2)	14.8 (1.1)	15.3 (1.4)	14.6 (1.3)	0.142
18:1n-7	1.2 (0.2)	1.3 (0.1)	1.2 (0.2)	1.2 (0.1)	0.128
18:2n-6	12.1 (1.1)	12.2 (1.6)[a]	11.3 (1.3)	10.4 (1.7)[b]	0.003
18:3n-3	0.2 (0.1)	0.2 (0.04)	0.2 (0.04)	0.17 (0.1)	0.144
20:3n-6	2.2 (0.6)	2.3 (1.0)[a]	2.0 (0.7)	1.7 (0.4)[b]	0.01
20:4n-6	17.3 (2.2)	17.1 (2.2)[a]	16.9 (2.9)[a]	15.1 (1.9)[b]	0.001
20:5n-3	1.1 (0.4)	1.1 (0.4)[a]	1.6 (0.6)[a]	4.2 (1.4)[b]	0.001
22:4n-6	3.6 (0.9)	3.6 (0.8)	3.4 (1.1)[a]	2.7 (0.9)[b]	0.003
22:5n-6	0.6 (0.1)	0.6 (0.1)[a]	0.6 (0.1)[b]	0.4 (0.1)[b]	0.001
22:5n-3	3.3 (0.6)	3.3 (0.6)[a]	3.1 (0.6)[b]	4.3 (0.4)[b]	0.001
22:6n-3	5.74 (1.53)	6.0 (1.3)[a]	7.7 (2.0)[b]	6.3 (1.2)	0.025

Mean values (SD)

P value is from univariate analysis of variance of the follow-up value by treatment with baseline value, ethnicity, BMI and age as covariates

Values in the same row with different superscripts are significantly different from each other P<0.05 using Bonferroni's test for 3 comparisons.

Two outliers were identified from the omega-3 index (erythrocyte EPA+DHA) measurement, used as an index of compliance (Figure 3-3). The omega-3 index increased from 4.3% to 9.4% for one subject in the placebo group, and decreased from 13.1% to 6.1% for another subject in the DHA group. The latter was an outlier at baseline for both EPA and DHA levels (2.7% and 10.4%, respectively), while EPA and/or DHA levels from the subject in the placebo group did not differ from the rest of the group at baseline and 6 weeks. Neither of these participants returned their capsules. Table 3-5 shows the erythrocyte lipids individually and in categories, excluding these participants. The omega-3 index increased by 2.7% (95% CI, +1.5, +4.0, $P = 0.009$) in the DHA group and 3.9% (95%CI, +2.7, +5.1, $P < 0.001$) in the EPA group. Treatment did not affect total SFA or total (*n*-3 + *n*-6*)* PUFA levels in erythrocytes. EPA but not DHA significantly reduced total *n*-6 PUFA by 5.29% (95% CI, -6.42, -4.16, $P < 0.001$, as well as individual *n*-6 PUFA such as LA and AA. Total *n*-3 were increased to a greater extent following EPA treatment (+4.76%, 95% CI, +3.17, +6.36, $P <0.001$) compared to DHA (+2.48%, 95%CI, +1.32, +3.65, $P = 0.019$). EPA levels were increased by EPA treatment by 3.03% (95% CI, +2.30, +3.78, $P < 0.001$) and to a lesser extent by DHA treatment (+0.59%, 95% CI, +0.29, +0.90, $P = 0.006$), while DHA was increased in the DHA group only (+2.12%; 95%CI, +1.14, +3.11, $P = 0.005$). EPA but not DHA increased DPA levels by +0.87% (95% CI, +0.43, +1.31, $P < 0.001$). *n*-6:*n*-3 PUFA ratio was reduced to a greater extent with EPA (-1.52%, 95% CI, -2.04, -1.00, $P < 0.001$) than DHA (-0.77%, 95% CI, -1.23, -0.31, $P = 0.013$).

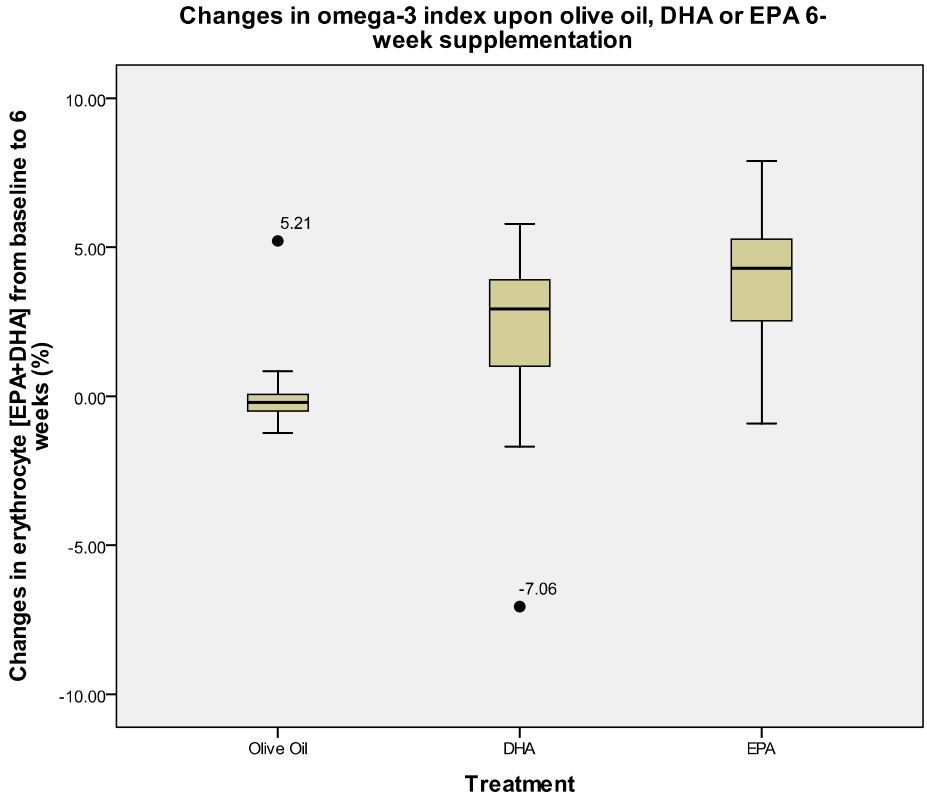

Figure 3-3 Effect of EPA and DHA supplementation on the omega-3 index

The bottom and top of the box represent the lower and upper quartiles, respectively, and the band near the middle of the box is the median. Error bars are 95% confidence intervals. Outliers are dots out of the error bars, representing an increase of the omega-3 index by +5.21% in the olive oil group, and a decrease by -7.06% in the DHA group.

Table 3-5: Erythrocyte fatty acid composition (%) at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3 g/d) after excluding 2 outliers

Fatty acids	Run-in (*n* = 46)	Olive Oil (*n* = 15)	DHA (*n* = 15)	EPA (*n* = 16)	*P* value
Total SFA	32.4 (3.5)	32.34(4.0)	33.0 (3.4)	33.9 (2.7)	0.098
16:0	17.0 (2.5)	17.0 (3.2)	17.4 (2.1)	18.3 (1.6)	0.179
18:0	15.3 (1.3)	15.1 (1.1)	15.5 (1.4)	15.6 (1.3)	0.394
Total MUFA	16.5 (1.2)	16.5 (1.0)[a]	16.5 (0.9)[a,b]	16.2 (1.4)[b]	**0.017**
16:1	0.3 (0.1)	0.4 (0.5)	0.3 (0.1)	0.3 (0.05)	0.987
18:1trans	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.1 (0.1)	0.490
18:1*n*-9	14.8 (1.2)	14.7 (1.2)[a]	15.1 (1.4)[a,b]	14.5 (1.3)[b]	**0.048**
18:1*n*-7	1.2 (0.1)	1.3 (0.1)	1.2 (0.2)	1.2 (0.1)	0.174
Total PUFA	45.61 (3.18)	46.2 (4.5)	45.8 (3.0)	45.1 (2.2)	0.550
Total *n*-6 PUFA	35.33 (2.95)	35.7 (4.7)[a]	33.0 (3.4)[a]	30.1 (3.7)[b]	**< 0.001**
18:2*n*-6	12.0 (1.1)	12.2 (1.6)[a]	11.0 (1.0)[a,b]	10.3 (1.7)[b]	**< 0.001**
20:3*n*-6	2.1 (0.6)	2.12 (1.1)[a]	1.8 (0.6)[a,b]	1.6 (0.4)[b]	**< 0.001**
20:4*n*-6	17.0 (2.0)	17.1 (2.2)[a]	16.3 (2.1)[a,b]	15.0 (1.8)[b]	**0.001**

Fatty acids	Run-in (*n* = 46)	Olive Oil (*n* = 15)	DHA (*n* = 15)	EPA (*n* = 16)	*P* value
22:4*n*-6	3.5 (0.8)	3.5 (0.9)	3.1 (0.9)	2.5 (0.9)	0.058
22:5*n*-6	0.5 (0.1)	0.6 (0.1)[a]	0.6 (0.1)[a]	0.4 (0.1)[b]	**< 0.001**
Total n-3 PUFA	10.1 (1.8)	10.3 (1.5)[a]	12.5 (2.3)[b]	14.6 (2.5)[c]	**< 0.001**
18:3*n*-3	0.2 (0.1)	0.2 (0.04)[a]	0.2 (0.04)[a,b]	0.2 (0.1)[b]	**0.042**
20:5*n*-3	1.0 (0.3)	1.1 (0.4)[a]	1.6 (0.6)[b]	3.9 (1.4)[c]	**< 0.001**
22:5*n*-3	3.3 (0.6)	3.2 (0.6)[a]	3.0 (0.5)[a]	4.23 (0.4)[b]	**< 0.001**
22:6*n*-3	5.5 (1.4)	5.7 (1.2)[a]	7.6 (1.9)[b]	6.2 (1.2)[a,b]	**0.007**
ω3 Index	6.6 (1.6)	6.8 (1.4)[a]	9.2 (2.4)[b]	10.1 (2.4)[b]	**< 0.001**
VLC *n*-3 PUFA	9.9 (1.8)	10.1 (1.5)[a]	12.3 (2.3)[b]	14.5 (2.5)[c]	**< 0.001**
n-6 : *n*-3 PUFA	3.5 (0.8)	3.5 (0.8)[a]	2.6 (0.8)[b]	2.1 (0.8)[c]	**< 0.001**
20:5*n*-3/20:4*n*-6	0.1 (0.02)	0.1 (0.03)[a]	0.1 (0.04)[b]	0.3 (0.1)[c]	**< 0.001**
22:6*n*-3/20:4*n*-6	0.3 (0.1)	0.3 (0.1)[a]	0.5 (0.1)[b]	0.4 (0.1)[b]	**0.002**

Values are geometric means (SD), expressed in mass percentage of total erythrocyte fatty acid composition. *P* value is from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates. Values in the same row with different superscripts are significicantly different from each other, P<0.05 using Bonferroni's test for 3 comparisons.

VLC *n*-3 PUFA, very long chain n-3 PUFA (= 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3)

3.2.2 Plasma NEFA fatty acid composition.

Table 3-6 shows the fatty acid composition of the NEFA. In contrast to the erythrocyte lipids, the proportion of long-chain polyunsaturated fatty acids was much lower in NEFA. Both EPA and DHA decreased the proportion of palmitoleic acid (16:1n-7) and EPA that of oleic acid (18:1n-9) compared with placebo. EPA but not DHA increased NEFA EPA by +2.6% (95% CI; +1.1, +4.1, $P < 0.001$) while both EPA and DHA increased DHA levels (+1.4%; 95% CI; +0.6, +2.2; $P < 0.001$ and +1.1; 95% CI, +0.3, +1.8, $P = 0.001$, respectively). EPA but not DHA increased DPA levels ($P = 0.018$). EPA+DHA percentage was increased by +4.9% (95% CI; +1.7, +6.3; $P <0.001$) by EPA and 1.3% (95% CI; +0.4, +2.3; $P = 0.021$) by DHA. EPA but not DHA decreased *n*-6:*n*-3 ratio. Neither EPA nor DHA influenced the proportion saturated fatty acids in NEFA. Changes in NEFA were more variable compared to changes in erythrocyte composition. In order to compare NEFA and erythrocyte FA patterns, Spearman's correlations were performed between the two FA fractions for the main FA categories and n-3 PUFA (Table 3-7).

Table 3-6: NEFA composition (%) at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

	Run-in (*n* = 46)	Olive Oil (*n* = 15)	DHA (*n* = 15)	EPA (*n* = 16)	*P* value
Total SFA	62.5 (10.7)	58.0 (9.2)	59.6 (12.3)	57.8 (13.4)	0.714
16:0	3.3 (0.9)	3.0 (0.7)	2.8 (1.0)	3.3 (1.0)	0.382
18:0	41.9 (6.4)	38.1(6.7)	39.9 (7.3)	38.5 (8.0)	0.630
Total MUFA	15.2 (5.0)	16.6 (3.6)	16.6 (4.6)	15.8 (5.0)	0.898
16:1	17.9 (9.9)	23.0 (8.2)[a]	18.3 (9.0)[b]	17.1 (8.5)[b]	**0.044**
18:1trans	2.3 (1.0)	2.2 (0.8)	2.2 (0.9)	2.6 (1.0)	0.889
18:1*n*-9	15.1 (9.3)	20.5 (8.1)[a]	15.9 (8.4)[a]	14.3 (7.7)[b]	**0.035**
18:1*n*-7	15.5 (4.7)	16.5 (3.8)	18.1 (6.9)	20.0 (9.3)	0.134
Total PUFA	12.2 (4.0)	13.6 (3.4)	13.5 (5.8)	13.3 (5.0)	0.971
Total *n*-6 PUFA	4.8 (3.9)	7.0 (3.5)	4.6 (5.1)	5.6 (4.2)	0.655
18:2*n*-6	0.2 (0.2)	0.2 (0.2)	0.2 (0.1)	0.2 (0.1)	0.409
20:3*n*-6	0.7(0.4)	0.6 (0.2)	0.8 (0.3)	0.5 (0.4)	0.280
20:4*n*-6	1.5 (0.9)	1.4(0.4)	1.9 (1.0)	1.8 (1.2)	0.077
22:4*n*-6	2.8 (2.1)	2.9 (3.0)	3.1 (3.7)	3.1 (2.9)	0.328

	Run-in (*n* = 46)	Olive Oil (*n* = 15)	DHA (*n* = 15)	EPA (*n* = 16)	*P* value
22:5*n*-6	0.3 (0.3)	0.2 (0.1)	0.3 (0.1)	0.3 (0.4)	0.086
Total n-3 PUFA	3.1 (1.2)	2.8 (0.9)[a]	4.0 (2.6)[a]	6.2 (5.6)[b]	**<0.001**
18:3*n*-3	1.7 (0.7)	1.5 (0.6)	1.6 (0.6)	1.8 (0.7)	0.066
20:5*n*-3	0.3 (0.1)	0.3 (0.3)[a]	0.5 (0.4)[a]	1.8 (2.9)[b]	**<0.001**
22:5*n*-3	0.2 (0.2)	0.2 (0.1)[a]	0.2 (0.3)[a]	0.6 (0.7)[b]	**0.004**
22:6*n*-3	0.8 (0.3)	0.7 (0.2)[a]	1.5 (1.6)[b]	1.7 (1.8)[b]	**<0.001**
EPA+DHA	1.1 (0.4)	1.1 (0.4)[a]	2.1 (2.0)[b]	3.4 (4.6)[b]	**<0.001**
VLC *n*-3 PUFA	1.3 (0.6)	1.3 (0.4)[a]	2.3 (2.2)[a]	4.0 (5.1)[b]	**<0.001**
n-6 : *n*-3 PUFA	3.9 (1.6)	4.9 (2.2)[a]	3.3 (2.6)[a,b]	2.1 (1.1)[b]	**0.001**
20:5*n*-3/20:4*n*-6	0.2 (0.2)	0.2 (0.1)[a]	0.3 (0.2)[a]	1.0 (1.1)[b]	**<0.001**
22:6*n*-3/20:4*n*-6	0.5 (0.4)	0.5 (0.2)[a]	0.8 (0.5)[b]	0.9 (0.6)[b]	**0.011**

Values are geometric means (SD), expressed in mass percentage of total NEFA composition. *P* value is from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates. Values in the same row with different superscripts are significantly different from each other, P<0.05 using Bonferroni's test for 3 comparisons.

VLC *n*-3 PUFA, very long chain n-3 PUFA (= 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3)

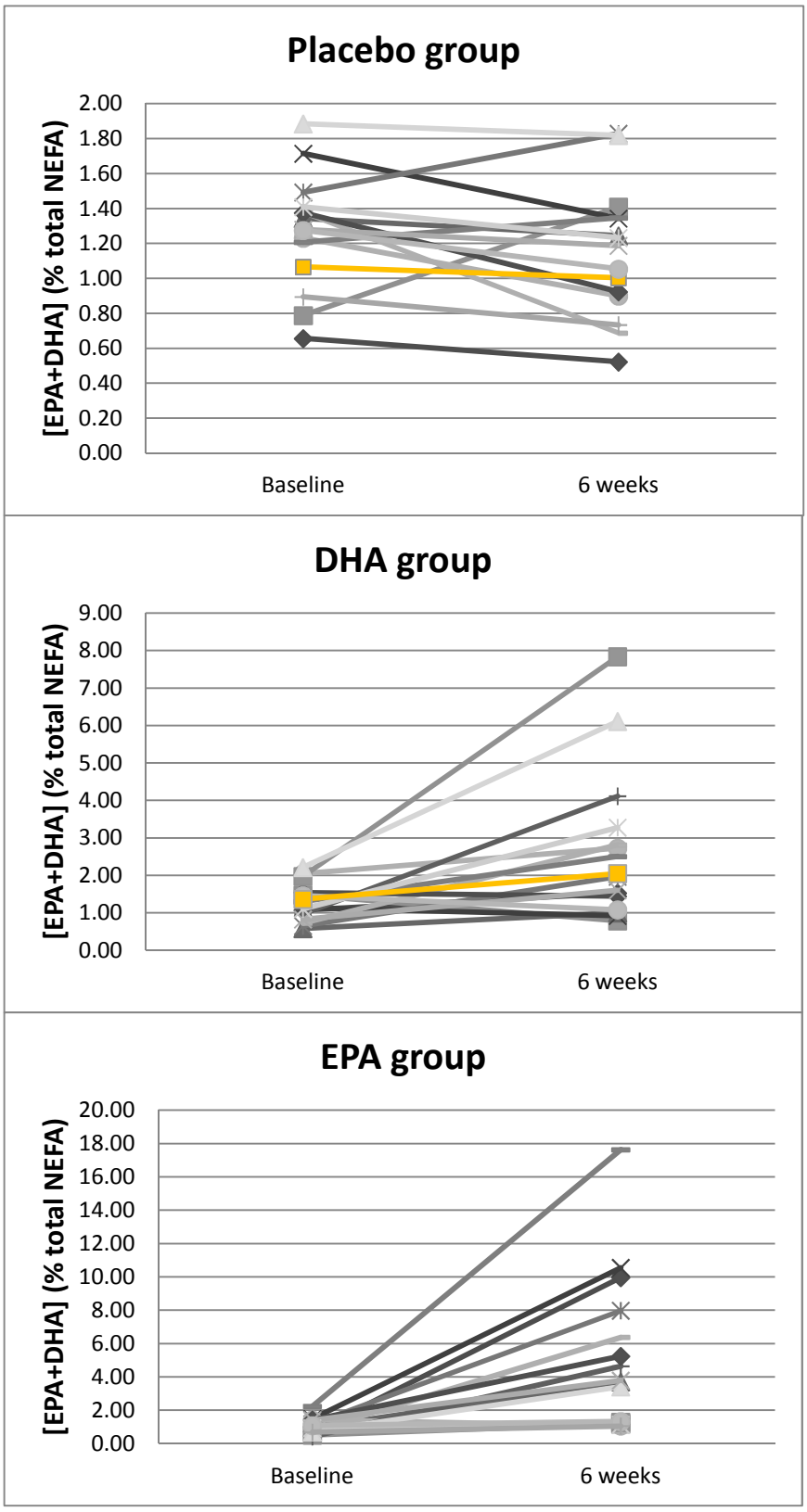

Figure 3-4: Changes in EPA+DHA in NEFA by treatmentComparison between erythrocyte lipids and NEFA profiles

Table 3-7 Pearson's bivariate correlation for the composition in the main FA categories and n-3 PUFA between erythrocyte and plasma NEFA at baseline and 6 weeks of intervention and for the changes form baseline.

	Baseline		6 weeks		Changes	
	r	*P*	*r*	*P*	*r*	*P*
SFA	0.153	0.310	-0.022	0.880	0.261	0.080
MUFA	0.259	0.093	-0.038	0.811	0.358*	0.025
PUFA	0.060	0.693	-0.128	0.387	-0.006	0.966
Total n-6 PUFA	0.057	0.705	-0.035	0.815	-0.172	0.252
Total n-3 PUFA	0.220	0.141	.404**	0.004	0.436**	0.002
ALA	0.320*	0.034	0.052	0.733	-0.172	0.275
EPA	0.044	0.769	0.508***	<0.001	0.527***	<0.001
DHA	0.384**	0.008	0.349*	0.015	0.284	0.056
DPA	0.189	0.203	0.354*	0.014	0.395**	0.006
EPA+DHA	0.326*	0.027	0.400**	0.005	0.406**	0.005
EPA+DHA+DPA	0.325*	0.028	0.434**	0.002	0.439**	0.002
n-6/n-3 ratio	0.264	0.076	0.362*	0.012	0.426**	0.003

Pearson's correlation coefficients are presented as r . Significant *P*-values (*P*) for correlation: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.2.3 EPC

Table 3-7 shows the EPC counts – relative to total mononuclear cells - at baseline and follow-up, as defined by the various combinations of bone marrow and endothelial markers described in Chapter 2. None of these counts, including early (CD34+/KDR+, CD133) and late (CD34+/CD31+/KDR+) EPC populations differ between (ANCOVA) or within groups (Wilcoxon matched-pairs signed rank tests). The reproducibility of the technique was assessed by Spearman correlation analysis between baseline and follow-up values.

Table 3-8: Endothelial circulating and progenitor cells at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d), in % of total mononuclear cells

		Olive oil *n* = 14	DHA *n* = 12	EPA *n* = 15	*P* value
CD34+/ CD31+	Baseline	0.45 (0.39)	0.52 (0.33)	0.49 (0.26)	
	6 weeks	0.44 (0.44)	0.66 (0.59)	0.55 (0.36)	
	Change	-0.01 (-0.19,0.17)	0.14 (-0.33,0.61)	0.06 (-0.15,0.26)	0.739
	r	0.434	0.429	0.393	
CD34+/ CD133+	Baseline	0.09 (0.10)	0.25 (0.30)	0.10 (0.13)	
	6 weeks	0.08 (0.08)	0.31 (0.34)	0.08 (0.11)	
	Change	-0.01 (-0.08,0.06)	0.05 (-0.12,0.22)	-0.02 (-0.11,0.07)	0.241
	r	0.452	0.750	-0.219	
CD34+/ KDR+	Baseline	0.20 (0.16)	0.26 (0.12)	0.34 (0.28)	
	6 weeks	0.14 (0.15)	0.28 (0.29)	0.28 (0.33)	
	Change	-0.06 (-0.20,0.08)	0.02 (-0.21,0.24)	-0.06 (-0.36,0.23)	0.382
	r	0.291	0.429	0.284	
KDR+/ CD133+	Baseline	0.07 (0.06)	0.25 (0.19)	0.07 (0.06)	

		Olive oil *n* = 14	DHA *n* = 12	EPA *n* = 15	*P* value
	6 weeks	0.07 (0.05)	0.26 (0.25)	0.09 (0.11)	
	Change	0.00 (-0.05,0.05)	0.01 (-0.18,0.20)	0.02 (-0.03,0.07)	0.592
	r	0.263	0.500	0.492	
CD34+/ CD133+/ KDR+ (Early EPC)	Baseline	0.05 (0.05)	0.17 (0.11)	0.05 (0.06)	
	6 weeks	0.05 (0.04)	0.21 (0.19)	0.06 (0.11)	
	Change	0.00 (-0.04,0.03)	0.04 (-0.13,0.22)	0.01 (-0.05,0.07)	0.147
	r	0.814	0.071	-0.093	0.410
CD31+/ KDR+/ CD34+ (Late EPC)	Baseline	0.17 (0.14)	0.22 (0.19)	0.22 (0.17)	
	6 weeks	0.14 (0.11)	0.20 (0.21)	0.31 (0.17)	
	Change	-0.03 (-0.14,0.08)	-0.02 (-0.26,0.21)	0.09 (-0.03,0.22)	0.081
	r	0.165	0.250	0.414	0.246

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). *P* values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates. *r* values are Spearman's correlation coefficients

3.2.4 Capillary density

Table 3-8 shows the results for capillary density. The values for capillary density are slightly lower than literature values. There were no significant changes on treatment and the method generally appeared to have good reproducibility as judged the correlation between baseline and follow-up measures.

Table 3-9: Capillary density (capillary count / mm^2) at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

		Olive Oil (n = 15)	DHA (n = 15)	EPA (n = 15)	P value
Capillary density area, %	Baseline	5.06 (0.86)	5.52 (0.71)	5.65 (1.30)	
	6 weeks	5.26 (1.06)	5.53 (1.06)	5.88 (1.25)	
	Change	0.20 (-0.25 ; 0.66)	0.01 (-0.72 ; 0.73)	0.24 (-0.30 ; 0.77)	0.889
	r	0.461	-0.042	0.665	
Capillary density count	Baseline	65.02 (8.57)	69.45 (6.51)	68.85 (12.02)	
	6 weeks	69.04 (9.35)	69.82 (11.76)	70.88 (11.96)	
	Change	4.02 (-1.70 , 9.74)	0.37 (-6.25 , 7.00)	2.03 (-1.76 , 5.83)	0.773
	r	0.377	0.227	0.843	

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). P values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates. Capillary density is expressed as area covered by the capillaries relative to the total skin area (%) or as the number of capillaries per surface unit (capillary count). r values are Spearman's correlation coefficients.

3.2.5 Vascular measurements: PWA, DVP

The indexes of vascular function measured by PWA and DVP did not differ at baseline and 6 weeks of intervention between the groups (Table 3-10), or within the groups (Paired t-tests).

Table 3-10: Vascular function parameters at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

		Olive Oil (n = 13)	DHA (n = 15)	EPA (n = 15)	P value
Stiffness index, *m/s*	Baseline	5.7 (0.4)	6.1 (1.6)	6.12 (0.8)	
	6 weeks	5.6 (0.6)	6.1 (1.7)	5.9 (0.5)	
	Change	-0.0 (-0.5 , 0.4)	-0.0 (-0.2 , 0.1)	-0.2 (-0.6 , 0.1)	0.706
Reflection index, %	Baseline	65.4 (11.9)	64.6 (11.7)	68.0 (11.3)	
	6 weeks	67.5 (11.8)	65.5 (14.7)	66.4 (12.8)	
	Change	0.8 (-8.8 , 10.3)	0.2 (-6.7 , 7.0)	-1.6 (-6.5 , 3.4)	0.797
Central SBP, *mmHg*	Baseline	101.6 (7.3)	101.7 (8.5)	101.1 (8.7)	
	6 weeks	99.4 (6.8)	101.9 (7.9)	100.2 (10.3)	
	Change	-2.7 (-7.2 , 1.8)	1.5 (-2.4 , 5.4)	-1.0 (-6.6 , 4.7)	0.633
Central DBP, *mmHg*	Baseline	65.5 (5.8)	65.8 (7.3)	65.1 (5.5)	
	6 weeks	64.0 (5.5)	66.0 (8. 5)	65.7 (9.12)	
	Change	-1.4 (-4.6 , 1.8)	0.6 (-2.8 , 4.0)	0.6(-3. 9 , 5.0)	0.503
Peripheral AIx, %	Baseline	54.0 (9.0)	50.0 (15.1)	53.7 (13.2)	
	6 weeks	52.7 (9.5)	48.9 (15.2)	50.2 (12.1)	
	Change	-2. 5 (-6.6 , 1.7)	0.3 (-3. 7 , 4.3)	-3.5 (-8.5 , 1.5)	0.645
Central AIx, %	Baseline	112.2 (8.3)	108.4 (11.0)	110. 6 (11.3)	
	6 weeks	111.0 (8.3)	105.5 (13.7)	107.7 (10.5)	
	Change	-2.1 (-6.9 , 2.7)	-2.6 (-6.1 , 0.9)	-2.9 (-8.7 , 2.8)	0.560

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). *P* values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates. AIx, Augmentation index (= P2/P1, where P1 and P2 are the pressure of the forward (systolic) and reflected (diastolic) wave, respectively.

3.2.6 BP and HR

3.2.6.1 Seated and supine BP and HR

Seated and supine SBP, DBP and HR did not differ between the groups at baseline and 6 weeks of intervention (**Table 3-12**). Paired t-tests showed no significant difference between end point values and baseline within each group (*P* values not shown).

Table 3-11: Seated SBP, DBP and HR at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

		Olive Oil (*n* = 16)	DHA (*n* = 16)	EPA (*n* = 16)	*P* value
SBP, *mmHg*	Baseline	120.8 (10.6)	121.6 (6.7)	117.8 (9.2)	
	6 weeks	118.8 (10.2)	120.6 (8.6)	115.5 (12.3)	
	Change	-2.0 (-4.4 ; 0.4)	-1.1 (-5.2 ; 3.1)	-2.3 (-6.8 ; 2.2)	0.902
DBP, *mmHg*	Baseline	67.0 (8.7)	69.2 (6.7)	68.6 (10.1)	
	6 weeks	65.6 (7.6)	68.2 (7.4)	65.9 (11.4)	
	Change	-1.4 (-4.1 ; 1.3)	-1.1 (-4.3 ; 2.2)	-2.7 (-5.7 ; 0.3)	0.875
HR, *bpm*	Baseline	63.4 (7.9)	66.3 (13.0)	65.9 (11.9)	
	6 weeks	64.8 (10.0)	65.3 (9.6)	65.3 (12.8)	
	Change	1.4 (-3.9 ; 6.8)	-0.9 (-7.4 ; 5.6)	-0.6 (-6.9 ; 5.7)	0.990

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). *P* values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates.

Table 3-12: Supine systolic, diastolic, mean arterial blood pressure (SBP, DBP, MAP) and supine heart rate (HR) at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

		Olive Oil (n = 16)	DHA (n = 16)	EPA (n = 16)	P value
SBP, *mmHg*	Baseline	118.0 (9.6)	119.8 (9.2)	118.8 (10.6)	
	6 weeks	116.3 (7.2)	121.5 (8.1)	119.0 (13.1)	
	Change	-1.7 (-6.6 ; 3.1)	1.7 (-3.0 ; 6.4)	0.2 (-7.3 ; 7.7)	0.625
DBP, *mmHg*	Baseline	64.3 (5.2)	64.5 (7.0)	64.2 (5.5)	
	6 weeks	63.1 (5.7)	65.2 (8.4)	64.5 (8.4)	
	Change	-1.2 (-4.0 ; 1.6)	0.7 (-2.7 ; 4.1)	0.2 (-3.6 ; 4.1)	0.517
MAP, *mmHg*	Baseline	80.8 (6.1)	80.7 (7.2)	81.4 (6.3)	
	6 weeks	80.8 (6.1)	80.7 (7.2)	81.4 (6.3)	
	Change	-1.5 (-4.7 ; 1.7)	0.6 (-3.1 ; 4.3)	-0.6 (-5.4 ; 4.1)	0.751
HR1, *bpm*	Baseline	55.4 (5.5)	57.9 (6.3)	56.8 (7.5)	
	6 weeks	56.1 (7.1)	56.3 (7.9)	56.9 (8.4)	
	Change	0.7 (-1.9 ; 3.2)	-1.5 (-5.4 ; 2.3)	0.1 (-4.1 ; 4.3)	0.824
HR2, *bpm*	Baseline	57.2 (6.2)	60.2 (7.0)	59.5 (7.5)	
	6 weeks	57.6 (7.1)	58.5 (7.5)	59.4 (7.4)	
	Change	0.4 (-3.6 ; 4.4)	-1.7 (-6.0 ; 2.6)	-0.1 (-3.6 ; 3.3)	0.816

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). *P* values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates. SBP, DBP, MAP and HR 1 were measured during the PWA assessment. HR 2 was measured during DVP analysis.

3.2.6.2 Ambulatory BP and HR

The evolutions of ambulatory BP and HR throughout the day are shown in

Figure 3-5 and Figure 3-6. 24h and daytime SBP, DBP, MAP and HR, as well as night SBP and HR did not differ at baseline (Table 3-13). Night DBP and MAP at baseline were higher in the EPA group compared to the placebo group ($P = 0.029$ and $P = 0.059$, respectively, Bonferroni's multiple comparison). There was no treatment effect on 24h and daytime SBP, DBP, MAP and HR, as well as night SBP, DBP and MAP (Table 3-13). At 6 weeks, night HR in the EPA and DHA group did not differ from the placebo group but was reduced by -6.5 bpm (95% CI; -12.2, -0.8) in the DHA group compared to the EPA group ($P = 0.020$) (Bonferroni multiple comparison). (Figure 3-7). The changes in night HR within the DHA group (-2.7 bpm; 95% CI; -0.1, -5.4) and in the EPA group (+3.0 bpm, 95%CI; -1.2, +7.2) did not reach significance ($P = 0.057$ and 0.140, respectively, paired t-tests). There was no significant difference within groups for the rest of ambulatory BP and HR values.

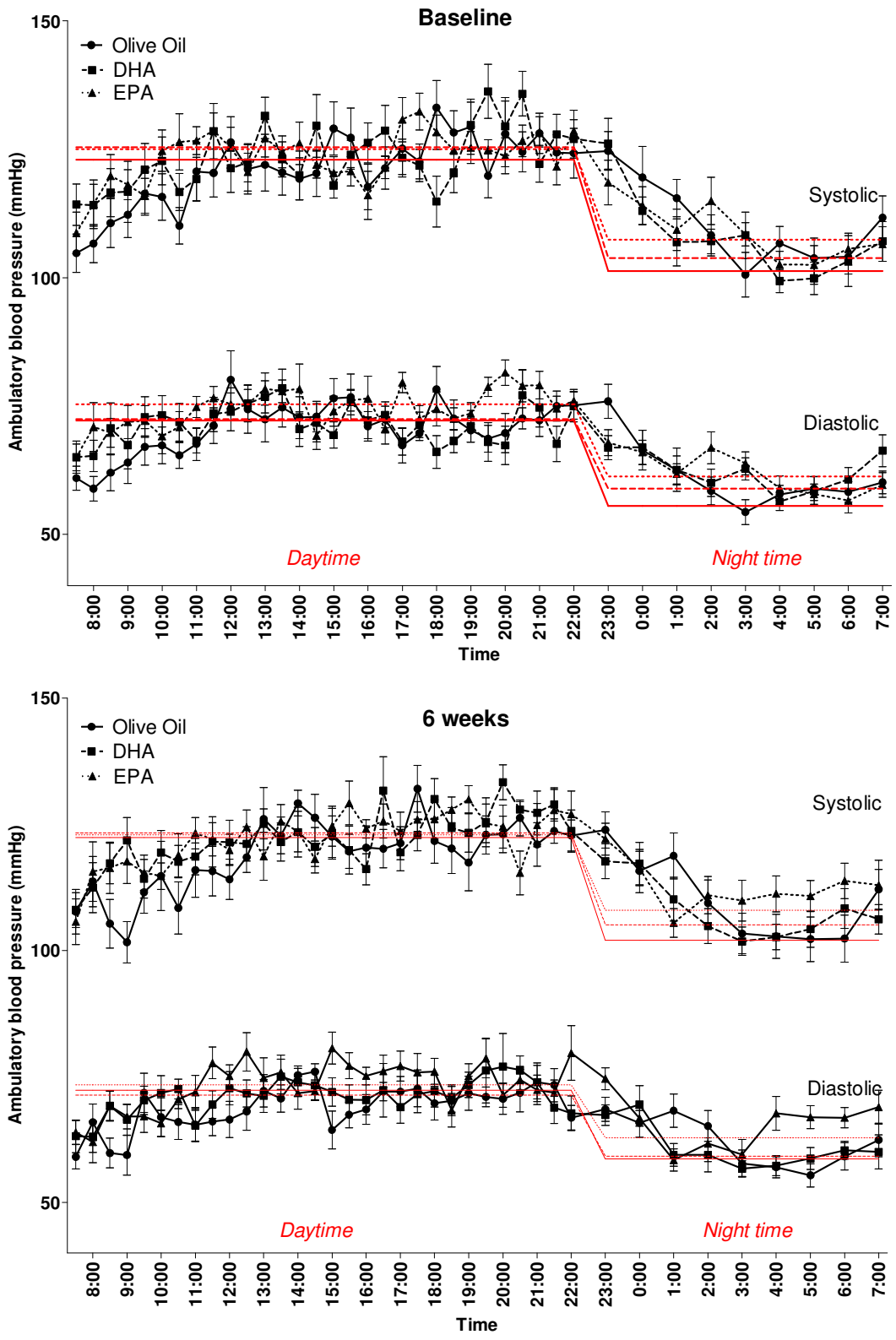

Figure 3-5: 24h systolic and diastolic blood pressure (BP) profiles at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

Values are mean BP measured every half an hour during the day (7am-10pm) and every hour at night time (10pm-7am), error bars are SEM. Red lines represent average daytime and night time BP measured for the placebo (solid), DHA (dashed) and EPA (dotted) groups.

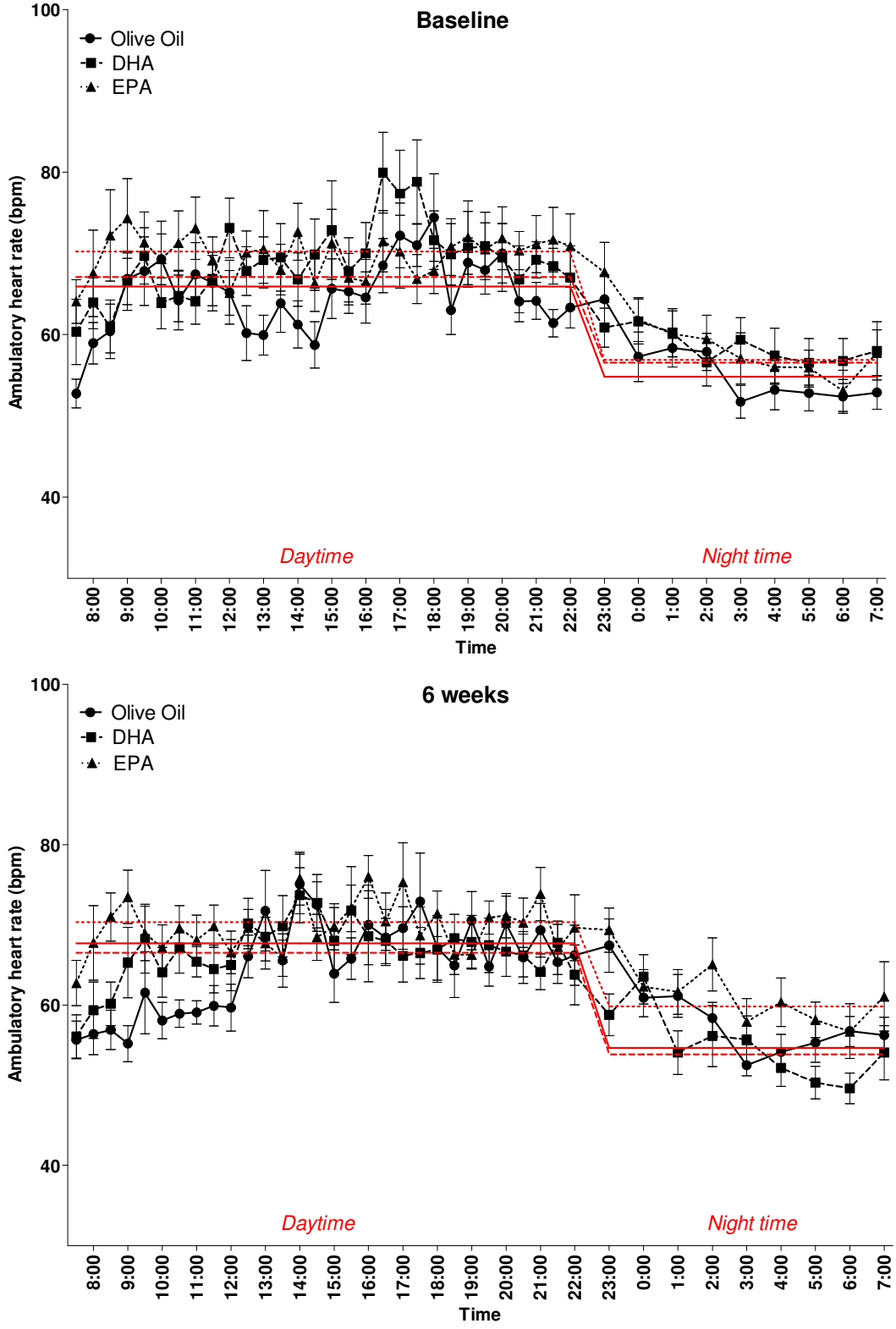

Figure 3-6: 24h heart rate (HR) profiles at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

Values are mean HR measured every half an hour during the day (7am-10pm) and every hour at night time (10pm-7am), error bars are SEM. Red lines represent average daytime and night time HR measured for the placebo (solid), DHA (dashed) and EPA (dotted) groups.

Table 3-13: 24h, daytime and nighttime ambulatory SBP, DBP, MAP and HR at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

			Olive Oil (n = 15)	DHA (n = 15)	EPA (n = 14)	P value
24h	SBP, *mmHg*	Baseline	117.4 (9.5)	120.5 (7.1)	121.0 (8.5)	
		6 weeks	116.0 (8.0)	118.4 (7.1)	119.4 (7.0)	
		Change	-1.4 (-5.3 ; 2.6)	-2.1 (-5.6 ; 1.5)	-1.6 (-5.3 ; 2.0)	0.663
	DBP, *mmHg*	Baseline	70.3 (10.7)	69.3 (6.9)	72.1 (4.8)	
		6 weeks	67.9 (5.2)	68.0 (5.4)	70.9 (4.4)	
		Change	-2.4 (-8.6 ; 3.9)	-1.3 (-3.2 ; 0.7)	-1.1 (-3.4 ; 1.1)	0.061
	MAP, *mmHg*	Baseline	83.9 (7.0)	86.1 (6.3)	88.0 (5.4)	
		6 weeks	83.6 (5.9)	84.4 (5.5)	86.8 (4.6)	
		Change	-0.3 (-3.2 ; 2.6)	-1.7 (-3.7 ; 0.3)	-1.2 (-3.5 ; 1.1)	0.338
	HR, *bpm*	Baseline	62.5 (6.7)	64.5 (8.5)	67.4 (9.9)	
		6 weeks	63.4 (5.4)	63.2 (7.8)	67.9 (6.6)	
		Change	0.9 (-3.2 ; 5.0)	-1.3 (-3.9 ; 1.2)	0.5 (-4.9 ; 5.9)	0.186
Day	SBP, *mmHg*	Baseline	122.8 (10.5)	125.2 (7.7)	124.9 (8.6)	
		6 weeks	122.4 (8.7)	123.3 (6.8)	122.9 (7.7)	
		Change	-0.5 (-4.2 ; 3.3)	-1.9 (-6.1 ; 2.2)	-1.9 (-6.1 ; 2.2)	0.859
	DBP, *mmHg*	Baseline	72.0 (6.8)	72.3 (7.8)	75.1 (5.3)	
		6 weeks	72.3 (5.6)	71.3 (5.3)	73.4 (4.7)	
		Change	0.3 (-2.9 ; 3.5)	-1.0 (-3.7 ; 1.7)	-1.8 (-4.2 ; 0.6)	0.643
	MAP, *mmHg*	Baseline	88.7 (7.8)	89.6 (7.0)	91.3 (5.9)	
		6 weeks	88.7 (6.1)	88.1 (5.2)	89.6 (5.0)	
		Change	0.0 (-3.1 ; 3.1)	-1.5 (-4.2 ; 1.2)	-1.7 (-4.4 ; 0.9)	0.671
	HR, *bpm*	Baseline	65.9 (7.7)	67.1 (9.0)	70.2 (10.9)	
		6 weeks	67.7 (7.9)	66.5 (8.0)	70.4 (7.2)	

			Olive Oil (n = 15)	DHA (n = 15)	EPA (n = 14)	P value
		Change	1.8 (-2.1 ; 5.8)	-0.5 (-3.8 ; 2.7)	0.1 (-5.9 ; 6.2)	0.580
Night	SBP, *mmHg*	Baseline	101.1 (8.3)	103.7 (8.0)	107.2 (10.8)	
		6 weeks	102.0 (11.1)	105.1 (8.2)	107.9 (5.8)	
		Change	0.9 (-3.8 ; 5.6)	1.4 (-3.7 ; 6.5)	0.7 (-4.5 ; 5.9)	0.246
	DBP, *mmHg*	Baseline	55.4 (4.7)	58.7 (6.4)	61.1 (3.8)	
		6 weeks	58.6 (8.6)	59.2 (5.5)	62.9 (4.6)	0.392
		Change	3.3 (-0.5 ; 7.0)	0.5 (-3.1 ; 4.1)	1.8 (-0.9 ; 4.5)	
	MAP, *mmHg*	Baseline	70.2 (5.3)	73.4 (6.1)	75.9 (5.7)	
		6 weeks	72.5 (9.0)	74.2 (5.6)	77.6 (4.7)	
		Change	2.4 (-1.5 ; 6.2)	0.8 (-2.6 ; 4.2)	1.6 (-1.4 ; 4.6)	0.551
	HR, *bpm*	Baseline	54.8 (5.4)	56.5 (8.0)	56.9 (7.0)	
		6 weeks	54.6 (5.2)	53.9 (8.8)	59.9 (6.7)	
		Change	-0.2 (-5.3 ; 4.9)	-2.7 (-5.4 ; 0.1)	3.0 (-1.2 ; 7.2)	**0.021**

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). *P* values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates.

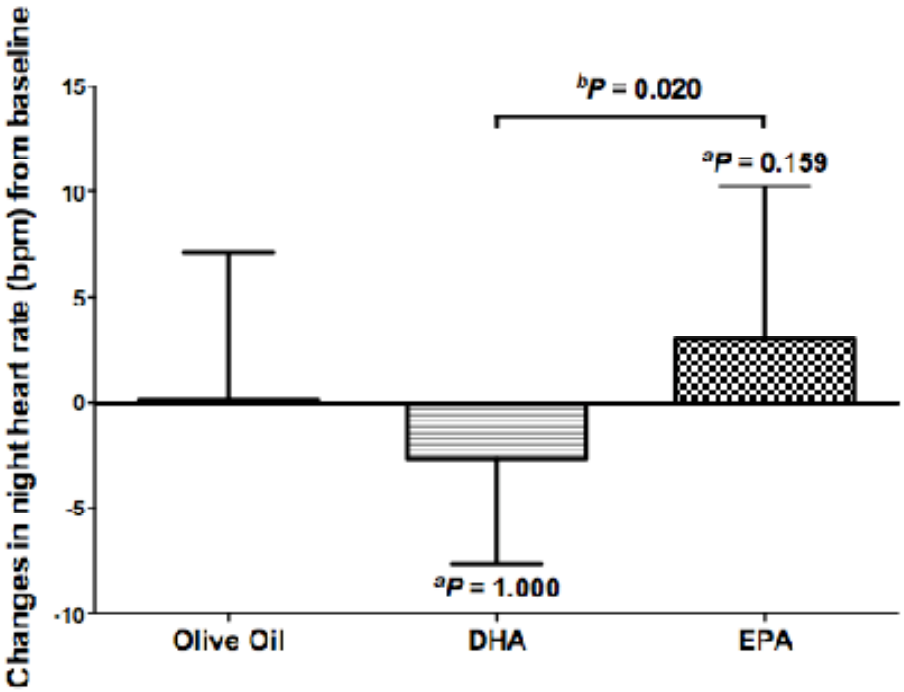

Figure 3-7: Changes in night heart rate from baseline to 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

Data are presented as means of the differences and errors bars are SD.

P values are from Bonferroni's multiple comparison with the placebo group (^{a}P) and between the EPA and DHA group (^{b}P) (*P* values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates).

3.2.6.3 Correlations with erythrocyte and NEFA very long chain (VLC) n-3 PUFA

In order to investigate whether there was any association between the changes observed in night heart rate and the incorporation of EPA and/or DHA in erythrocytes and NEFA, a partial correlation analysis was performed on the changes and on the endpoint values between night HR and EPA, DHA, EPA+DHA, DPA, EPA+DPA+DHA in erythrocytes and NEFA. There was no correlation of the changes but erythrocyte DPA were strongly correlated with night HR at 6 weeks (partial r = 0.443, P = 0.004) (partial correlation analysis, adjusted for age, BMI and ethnicity).

3.2.7 Nitrate and Nitrites (NOx)

There was no difference in NO levels at baseline and after 6 weeks of intervention (Figure 3-8); and there was no difference within group (Paired t-test).

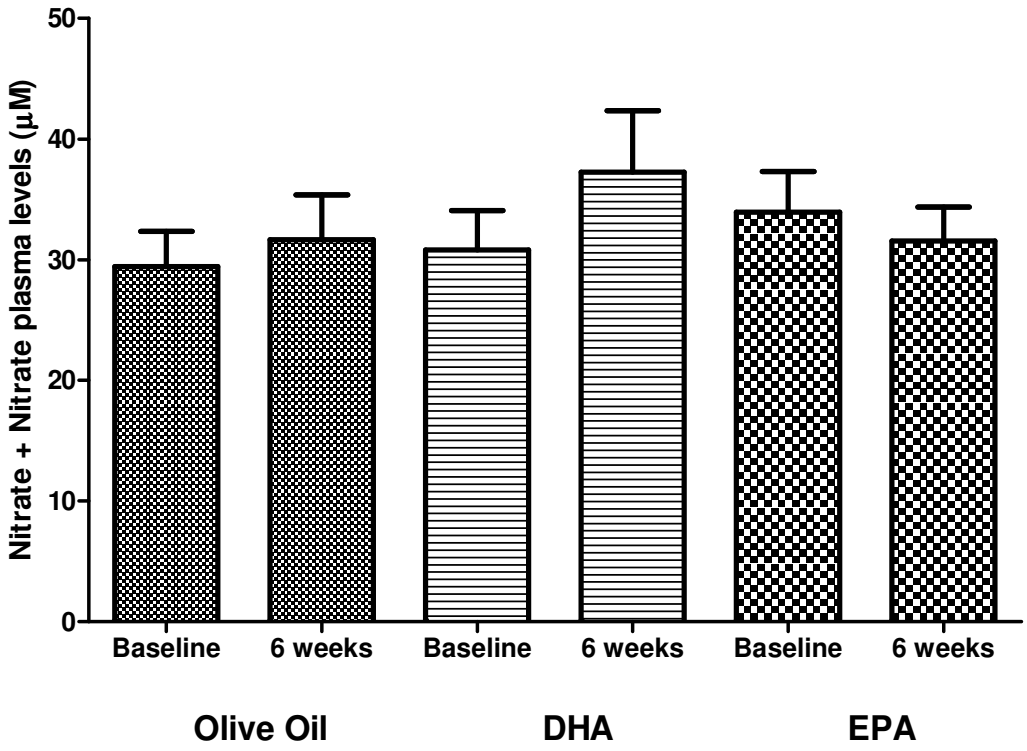

Figure 3-8: Total plasma nitrate and nitrite at baseline and 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

Data are presented as means and errors bars are SEM.

There was no difference between the groups (univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates) and within groups (Paired t-tests).

3.2.8 Isoprostanes

There was no difference between the groups in isoprostane levels at baseline and at 6 weeks of intervention. There was no within group difference (Wilcoxon matched-pairs signed rank test) (Figure 3-9). There was no significant correlation between isoprostane levels and VLC *n*-3 PUFA, in NEFA or erythrocyte at baseline (Pearson's bivariate correlation analysis).

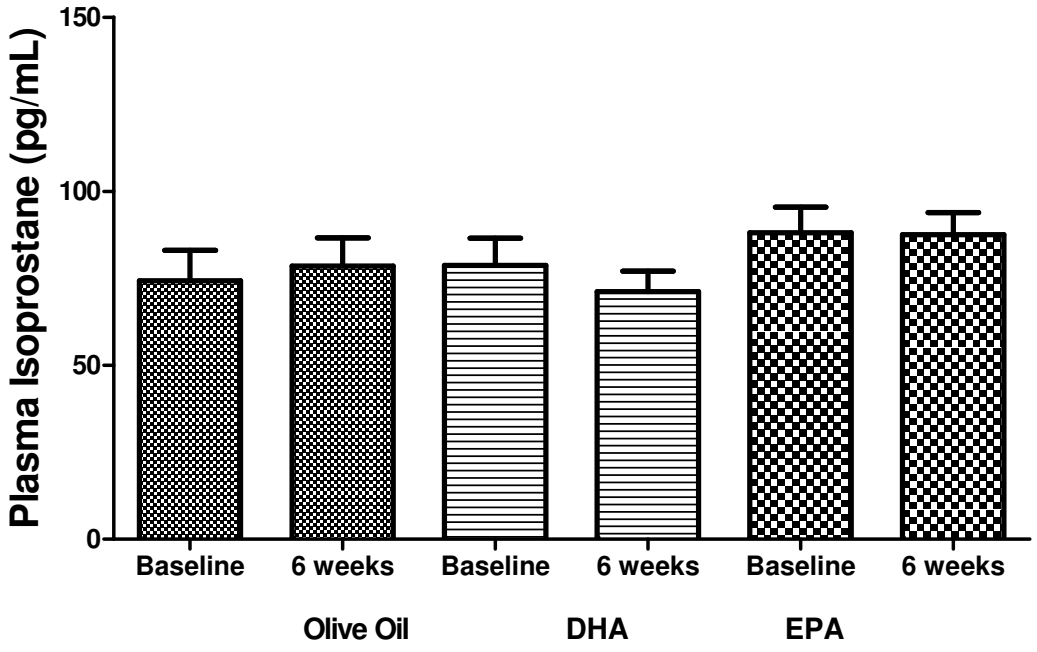

Figure 3-9: Plasma isoprostane at baseline and 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

Data are presented as means and errors bars are SEM.

There was no difference between the groups ($P = 0.261$, univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates) and within groups (Wilcoxon matched-pairs signed rank test).

3.2.9 Platelet Monocyte Aggregates (PMA)

Monocyte levels, platelet levels and platelet-monocyte aggregates (PMA) did not differ between the groups at baseline and six weeks of intervention (

Table **3-14**). There was no change within the groups throughout the study (paired t-tests). There was no correlation between the PMA and erythrocyte or NEFA VLC n-3 PUFA, taken together or individually (Pearson's bivariate correlation analysis).

Table 3-14: Platelet monocyte aggregates (PMA) at baseline and after 6 weeks of supplementation with placebo (olive oil), EPA or DHA (3g/d)

		Olive oil	DHA	EPA	*P* value
PMA (% total cells)	Baseline	0.58 (0.18)	0.44 (0.12)	0.59 (0.33)	
	6 weeks	0.59 (0.15)	0.49 (0.13)	0.62 (0.30)	
	Change	0.02 (-0.08; 0.11)	0.06 (-0.03; 0.14)	0.03 (-0.08; 0.14)	0.318
Monocytes (% total cells)	Baseline	6.08 (1.67)	5.49 (1.43)	7.09 (2.12)	
	6 weeks	6.72 (1.90)	5.95 (1.45)	7.42 (1.41)	
	Change	0.63 (-0.06; 1.32)	0.46 (-0.39; 1.30)	0.34 (-0.69; 1.36)	0.485
PMA (% aggregated monocytes)	Baseline	9.55 (1.78)	8.21 (2.17)	7.86 (2.64)	
	6 weeks	9.08 (1.45)	8.49 (2.05)	8.26 (2.69)	
	Change	-0.48 (-2.00; 1.04)	0.28 (-1.48; 2.04)	0.40 (-0.82; 1.62)	0.917
Platelets (% total cells)	Baseline	13.95 (2.72)	13.59 (4.52)	15.46 (10.31)	
	6 weeks	14.18 (3.57)	14.98 (5.10)	14.67 (7.27)	
	Change	0.23 (-1.62; 2.07)	1.39 (-0.72; 3.51)	-0.79 (-7.06; 5.48)	0.643

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). *P* values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates.

3.2.10 Plasma lipid profiles

Baseline lipid levels did not differ between groups at baseline. Neither EPA nor DHA affected plasma lipid concentrations at 6 weeks of intervention, including TAG, total cholesterol, LDL cholesterol and HDL cholesterol compared to placebo. Total cholesterol/HDL cholesterol and TAG/HDL cholesterol ratios also remained unchanged throughout the 6-week intervention with EPA or DHA compared to placebo (Table 3-15). There was a borderline significant effect of treatment on total NEFA concentrations (P = 0.048). Post hoc multiple comparison test showed NEFA levels were reduced by 124μmol/L (95%CI; -251, +4) following EPA (P = 0.061) and by 99μmol/L (95%CI; -226, +29) following DHA (P = 0.180) compared to placebo.

Paired t-tests comparing baseline and 6 week plasma lipid concentrations showed TAG levels decreased by -27 mM in the EPA group (P = 0.005) while they decreased by -13 mM in the placebo group and -14 mM in the DHA group without reaching significance (P = 0.109 and 0.221, respectively). The variations observed in total cholesterol, LDL and HDL cholesterol were not significant in all groups. Total cholesterol / HDL ratio decreased by 0.24 (-7.2 %) in the EPA group (P = 0.013) but not in the placebo and DHA groups. TAG/HDL ratio was significantly reduced in the EPA group by 0.21 (-30.3%, P = 0.007) while it decreased by 0.14 (-20.9%) in the DHA group without reaching significance (P = 0.072). In the EPA group, apolipoprotein B levels and ApoB/LDL ratio were slightly but significantly decreased by -6.3 (-6.7%, P = 0.044) and -4.7 (-4.8%, P = 0.042) respectively, while they remained unchanged in the two other groups. Confidence intervals for the changes and P values for t-tests are given in Table 3-15.

To investigate whether there was any association between the effect observed on TAGs and total NEFA, and the incorporation of EPA and/or DHA into the erythrocytes and NEFA fraction, a partial correlation analysis was performed on the changes and endpoint values of TAGs and total plasma NEFA, with EPA, DHA, EPA+DHA, DPA, EPA+DPA+DHA in erythrocytes and NEFA..Changes in TAG were inversely correlated with changes in the erythrocyte omega-3 index (partial r = -0.333, P = 0.029) and erythrocyte EPA (partial r = -0.367, P = 0.014) but not erythrocyte DHA alone (P = 0.209). Changes in NEFA were inversely correlated to the changes in omega-3 index (partial r = -0.444, P = 0.003), erythrocyte DHA (partial r = -0.370, P = 0.015) and EPA (partial r = -0.343, P = 0.022) as well as NEFA DHA (partial r = -0.338, P = 0.023) (partial correlation analysis, adjusted for age, BMI and ethnicity).

Table 3-15: Triglyceride (TAG), total cholesterol (TC), LDL and HDL levels, TC:HDL ratio and TAG:HDL ratio at baseline and after 6 weeks of supplementation with placebo (olive oil), DHA or EPA (3g/d).

		Olive Oil	DHA	EPA	Treatment effect §
TC, *mmol/L*	Baseline	4.8 (0.7)	4.5 (0.7)	4.7 (1.0)	
	6 weeks	4.7 (0.7)	4.6 (0.9)	4.7 (0.9)	
	Change	-0.1 (-0.5 , 0.3)	0.2 (-0.2 , 0.5)	0.0 (-0.4 , 0.4)	*P* = 0.673
	Paired t-test	NS	NS	NS	
TAG, *mmol/L*	Baseline	0.9 (0.3)	0.9 (0.4)	1.0 (0.5)	
	6 weeks	0.8 (0.3)	0.8 (0.3)	0.7 (0.3)	
	Change	-0.1 (-0.3 , 0.0)	-0.1 (-0.3 , 0.1)	-0.3 (-0.5 , -0.1)	*P* = 0.396
	Paired t-test	NS	NS	*P* = 0.005	
HDL*, *mmol/L*	Baseline	1.5 (0.4)	1.4 (0.3)	1.5 (0.3)	
	6 weeks	1.5 (0.4)	1.4 (0.3)	1.6 (0.5)	
	Change (ratio)	0.0 (-0.2 , 0.2)	0.06 (-0.0 , 0.2)	0.2 (-0.0 , 0.4)	*P* = 0.680
	Paired t-test	NS	NS	NS	
LDL, *mmol/L*	Baseline	2.9 (0.6)	2.7 (0.5)	2.8 (0.8)	

		Olive Oil	DHA	EPA	Treatment effect §
	6 weeks	2.8 (0.5)	2.9 (0.7)	2.8 (0.7)	
	Change	-0.1 (-0.3 , 0.2)	0.2 (-0.1 , 0.4)	-0.0 (-0.3 , 0.2)	*P* = 0.404
	Paired t-test	NS	NS	NS	
NEFA, *mmol/L*	Baseline	0.3 (0.1)	0.4 (0.2)	0.4 (0.0)	
	6 weeks	0.4 (0.2)	0.3 (0.1)	0.3 (0.0)	
	Change	0.1 (-0.0 , 0.2)	-0.1 (-0.2 , 0.0)	-0.1 (-0.2 , 0.0)	***P* = 0.048**
	Paired t-test	NS	NS	NS	
TC:HDL, *molar ratio*	Baseline	3.3 (0.8)	3.4 (0.8)	3.3 (0.6)	
	6 weeks	3.2 (0.7)	3.3 (0.7)	3.1 (0.7)	
	Change	-0.1 (-0.4 , 0.1)	-0.1 (-0.3 , 0.1)	-0.2 (-0.4 , -0.1)	*P* = 0.367
	Paired t-test	NS	NS	*P* = 0.013	
TAG:HDL, *molar ratio*	Baseline	0.7 (0.4)	0.7 (0.4)	0.7 (0.4)	
	6 weeks	0.6 (0.4)	0.5 (0.2)	0.5 (0.3)	
	Change	-0.1 (-0.2 , 0.0)	-0.1 (-0.3 , 0.0)	-0.2 (-0.4 , -0.1)	*P* = 0.442

		Olive Oil	DHA	EPA	Treatment effect §
	Paired t-test	NS	NS	*P* = 0.007	
ApoB, *g/L*	Baseline	0.9 (0.2)	0.8 (0.3)	0.9 (0.2)	
	6 weeks	0.8 (0.2)	0.9 (0.2)	0.9 (0.2)	
	Change	-0.1 (-0.2 , 0.1)	0.1 (-0.1 , 0.2)	-0.1 (-0.1 , -0.0)	*P* = 0.264
	Paired t-test	NS	NS	*P* = 0.044	
ApoB/LDL, *mass ratio*	Baseline	0.8 (0.1)	0.8 (0.2)	0.8 (0.1)	
	6 weeks	0.8 (0.2)	0.8 (0.1)	0.8 (0.1)	
	Change	-0.0 (-0.1 , 0.1)	0.0 (-0.1 , 0.1)	-0.0 (-0.1 , 0.0)	*P* = 0.632
	Paired t-test	NS	NS	*P* = 0.042	

Data are presented as means (SD) and changes from baseline are differences between the means (CI 95%) at baseline and six weeks. * Data are geometric mean (SD) and changes are expressed as ratio of change from baseline to 6 weeks

§ P values are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates.

NS, non significant (P > 0.05)

3.2.11 Glycaemic control

Treatment did not affect glucose and insulin levels, HOMA-IR or QUICKI at the end of the intervention (Table 3-16). Within group analysis showed no significant changes in fasting glucose, insulin, HOMA-IR and QUICKI, in any of the groups.

Table 3-16: Plasma glucose and insulin levels, HOMA-IR and QUICKI at baseline and after 6 weeks of supplementation with placebo (olive oil), DHA or EPA (3g/d).

		Olive Oil	DHA	EPA	P-values
Glucose, *mM*	Baseline	5.0 (0.3)	5.3 (0.3)	5.2 (0.4)	
	6 weeks	5.1 (0.4)	5.2 (0.5)	5.2 (0.8)	
	Change	0.1 (-0.2 , 0.4)	-0.0 (-0.2 , 0.2)	-0.0 (-0.4 , 0.3)	0.935
Insulin[1], *mU/L*	Baseline	5.4 (3.1)	6.4 (5.1)	5.6 (2.2)	
	6 weeks	6.7 (3.6)	6.7 (5.1)	6.1 (3.7)	
	Change	1.4 (-0.3 , 3.1)	0.3 (-1.3 , 1.9)	0.8 (-1.3 , 3.0)	0.826
HOMA-IR[1]	Baseline	1.2 (0.7)	1.5 (1.2)	1.3 (0.6)	
	6 weeks	1.5 (0.9)	1.6 (1.2)	1.4 (1.0)	
	Change	0.4 (-0.1 , 0.8)	0.1 (-0.3 , 0.4)	0.2 (-0.3 , 0.7)	0.775
QUICKI	Baseline	0.38 (0.03)	0.36 (0.03)	0.37 (0.03)	
	6 weeks	0.36 (0.03)	0.36 (0.03)	0.37 (0.03)	
	Change	-0.01 (-0.03, 0.00)	-0.00 (-0.01, 0.01)	-0.00 (-0.02, 0.01)	0.735

Data are presented as means (SD) and changes from baseline are expressed as mean of the differences (CI 95%). *P* values at 6 weeks are from univariate analysis of variance of the follow-up values by treatment with baseline values, ethnicity, BMI and age as covariates.[1] Values are geometric means (SD)

3.3 Discussion

The EPA and DHA trial (EDT) was designed to test whether EPA and DHA differentially improved endothelial and microvascular function in healthy young males, as assessed by the novel techniques of EPC and capillary density measurements, respectively. Neither EPA nor DHA affected EPC or capillary density, improved vascular function (arterial stiffness, vascular tone) or BP, but DHA and EPA lowered and increased ambulatory HR at night, respectively, and night time HR was directly correlated with erythrocyte DPA levels at the end of the intervention. Both EPA and DHA tended to decrease NEFA compared to placebo. Lipoprotein profiles were improved in the EPA group but the changes did not differ from the placebo and DHA groups. Neither DHA nor EPA affected other markers of endothelial function (NOx), oxidative stress (isoprostanes), platelet aggregation (PMA) or the indices of glycaemic control.

3.3.1 Erythrocyte lipids and NEFA profiles

3.3.1.1 The omega-3 index

Only two thirds of the participants (32 subjects) returned the capsules, of which 29 were identified as good compliers. Nonetheless the omega-3 index was increased by ~40% and ~60% in the DHA and EPA group, respectively, which suggests a good compliance to capsule intake. In addition to being an index of compliance, the omega-3 index has been proposed as an independent risk factor for cardiovascular risk, especially death from CHD (Harris and von Schacky, 2004). Percentages of EPA and DHA in erythrocytes at baseline were 1.1 and 5.7%, respectively, leading to an omega-3 index of 6.6%, which is similar to data previously reported in healthy males (Brown, et al., 1991, Sanders, et al., 1981, Sanders, et al., 2011) and corresponds to an intermediate risk of CHD according to the scale described by Harris and Von Schacky in 2004 (Harris and von Schacky, 2004). The omega-3 index remained stable in the olive oil group and increased to 9.2% and 10.1% in the DHA and EPA group, respectively, rising above the 8% target suggested by the authors to reach protection against death from CHD (). Although 4 to 6 months are needed to reach a steady state for the omega-3 index (Harris, 2010, Katan, et al., 1997), our findings confirm that the omega-3 index can be easily improved within a few weeks of fish oil supplementation in healthy subjects (Harris, 2010, Harris, et al., 2008, Larson, et al., 2008). Here we show that

either a high-EPA or high-DHA triglyceride oil (3g/d, 6 weeks) is sufficient to significantly raise the omega-3 index in healthy young males to a comparable extent, in order to reach the 8% desirable value. This is consistent with the only study – to our knowledge – that compared the effect of fish oils between EPA and DHA on erythrocyte lipid patterns, showing and increase in the omega-3 index from in elderly after 6 months of supplementation with 1.5g/d of either EPA or DHA (Vu, et al., 2007).

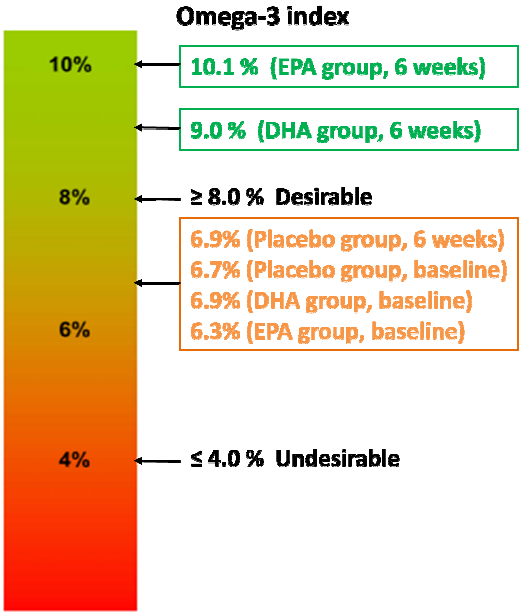

Figure 3-10: Omega-3 index in the placebo, DHA and EPA groups at baseline and 6 weeks of intervention in relation to the omega-3 index cut-points proposed by Harris and Von Schacky (Harris and von Schacky, 2004)

Values are geometric means; CV, cardiovascular.

3.3.1.2 Overall profiles

Erythrocyte lipid profiles were consistent with previous data (Hallund, et al., 2010, Milte, et al., Poppitt, et al., 2005, Viviani Anselmi, et al., 2010). However SFA in NEFA were slightly higher (~63%) compared to patterns previously observed, apparently at the expense of MUFA (~18%), while PUFA levels were consistent with previous data (Conquer, et al., 2002, Conquer, et al., 1999, Conquer and Holub, 1998, Frape, et al., 2000, Newens, et al., 2011, Rhee, et al., 2008). Little is known regarding the strength of the relationship between FA intake and fasting NEFA patterns, as NEFA may derive from the TAG stored in adipose tissue or present in the circulation (Arab, 2003). A high consumption of SFA prior to the visits or chronically would increase SFA in circulating and adipose tissue TAG, respectively, and both could affect fasting NEFA

composition. The high SFA levels in NEFA could also be due to a high endogenous supply (Sun, et al., 2007).

3.3.1.3 VLC *n*-3 PUFA in erythrocytes and NEFA

The changes of EPA, DPA and DHA levels in erythrocytes and NEFA throughout the 6 week intervention are reviewed in . Consistent with previous data, EPA was increased to a greater extent in the EPA group than DHA in the DHA group. Although the differential effect of EPA and DHA intake on erythrocyte or NEFA composition has been poorly investigated, there is substantial evidence that EPA is incorporated into erythrocyte and NEFA to a greater extent than DHA when they are given as a combination of both FA, either in the form of fish oils or oily fish (Cao, et al., 2006, Conquer, et al., 2002, Hallund, et al., 2010, Katan, et al., 1997, Pedersen, et al., 2010); and it is now well established that DHA turnover in erythrocyte membrane is slower than those of EPA (Brown, et al., 1991, Katan, et al., 1997). In erythrocytes, EPA supplementation increased EPA and DPA but not DHA, while DHA increased both EPA and DHA but not DPA (+ 0.6% and +2.1%, respectively), as previously observed in various populations and various lipid fractions, including plasma and platelet phospholipids (Hansen, et al., 1998, Kew, et al., 2004, Mori, et al., 2000). The EPA content of DHA capsules (8%, i.e. 0.4g / day) may have accounted for the EPA increase in the DHA group. However, the process of retroconversion from DHA to EPA is well documented and is more likely to explain the EPA increase (George, et al., 2007, Mori, et al., 2000, von Schacky and Weber, 1985). Our erythrocyte results also confirm that the elongation of EPA beyond DPA in humans is limited (Burdge, et al., 2002, Burdge and Wootton, 2002).

In NEFA, elongation from EPA to DPA was also observed in the EPA group, but DHA increased EPA levels in NEFA without reaching significance, suggesting retroconversion is limited in the NEFA fraction. EPA supplementation increased DHA NEFA levels, even to a greater extent than in the DHA group. Few studies have reported enrichment in DHA after EPA consumption in LDL fractions (Egert, et al., 2009), but this appears to be rare and relatively limited (Augoustides, et al., 2007, Gerster, 1998). The effect of supplementation of fish oils on fasting NEFA patterns has been poorly studied. In contrast with our findings, it was shown that EPA is preferentially incorporated into NEFA and DHA in TAG, a phenomenon only seen in non E4 carriers (Sung, et al., 2007). In addition, DHA was shown to be cleared from the chylomicrons at faster rate than EPA and it was suggested that DHA may be

preferentially cleaved by lipoprotein lipase to be transported – via NEFA - to the central nervous system, selectively enriched in DHA (Hansen, et al., 1998). Taken together, this suggests that EPA and DHA may be preferentially directed to different lipid fractions, which could play an important role in their differential effect on diverse CVD risk factors.

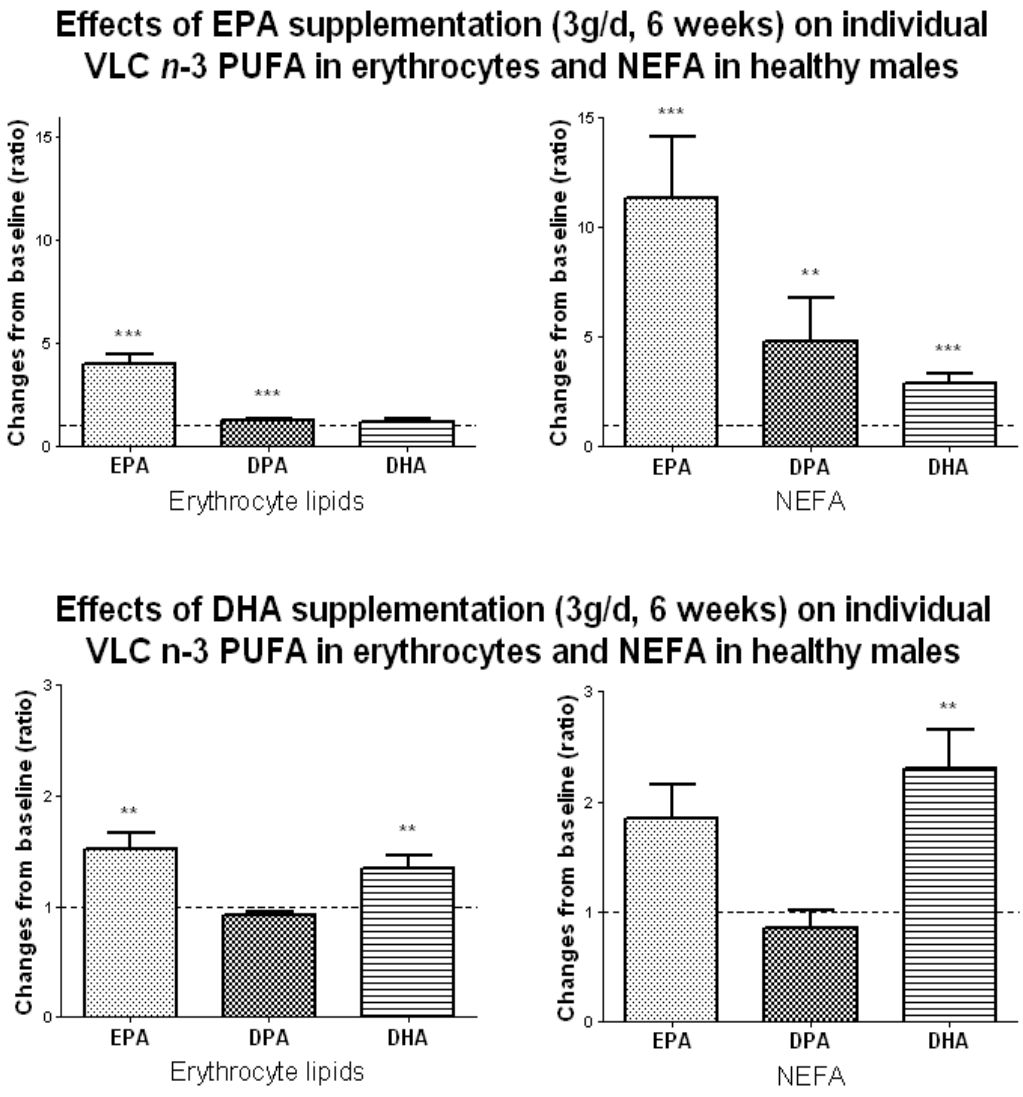

Figure 3-11: Changes in EPA, DPA and DHA in erythrocytes and NEFA upon EPA and DHA supplementation (3g/d, 6 weeks)

** $P < 0.01$, *** $P < 0.001$, values are significantly different from placebo (univariate analysis of variance of the follow-up value by treatment with baseline value, BMI and age as covariates)

3.3.2 Endothelial function: EPC and NOx

Despite the growing interest in EPC research over the past decade, their exact characterisation is still debated and there is to date no standardised method for a reproducible enumeration that could be used in clinical settings (Resch, et al., 2011), and results vary greatly from one study to another (Shaffer, et al., 2006, Werner, et al., 2005). Most studies have utilized flow cytometry and focused on the expression of the

stem cell markers CD133 and CD34, as well as endothelial markers KDR and CD31 (Nguyen, et al., 2012, Snedeker, et al., 2012). Here we have defined EPC as triple positive events representing two states of maturation of the EPC. Both early (CD34+/KDR+/CD133+) and late EPC (CD34+/KDR+/CD31+), as well as CD34+/KDR+ populations – also previously defined as EPC population - were higher than what we would expect in a healthy population (Egan, et al., 2008, Nguyen, et al., 2012, Peichev, et al., 2000, Shaffer, et al., 2006, Werner, et al., 2005). The present method counted stained cells in whole blood, which differs from the previously used Ficoll separation technique. The latter is a method of centrifugation used to isolate the mononuclear cells, resulting in a substantially loss of cells, which may explain this difference. The major limitation of this technique is the low EPC count, which restricted the reliability of the technique, as shown by the Pearson's correlation coefficients between baseline and follow-up for each group. These technical issues, and possibly individual variability may have blunted any effect of fish oils on EPC levels.

The phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB/Akt)/eNOS pathway plays a pivotal role in the process of EPC mobilization from the bone marrow to the circulation (Everaert, et al., 2010), and n-3 PUFA have been implicated in the modulation of this pathway, improving insulin signalling in endothelial cells and vascular inflammation (Delarue, et al., 2004, Schaefer, et al., 2008). It was hypothesised that fish oils would improve EPC levels through the improvement of NO production (Li, et al., 2007, Li, et al., 2007, Xue, et al., 2006), as well as oxidative stress (An, et al., 2009, McAnulty, et al., 2010), to which EPC are highly sensitive (Fleissner and Thum, 2011, Tilling, et al., 2009). Very little is known regarding the impact of FA on the novel biomarkers of EF including EPC and circulating progenitor cells (CPC), as well as those of endothelial damage, such as endothelial microparticle (MP) and and circulating endothelial cells (CEC) (Vafeiadou, et al., 2012). Wong *et al.* recently reported that fish oil supplementation for 12 weeks (4 g/d) improved renal function without affecting EPC, defined as CD133+/KDR+, or FMD in type 2 diabetics (Wong, et al., 2010). *n*-3 PUFA (5.4 g/d, 12 weeks) reduced platelet- and monocyte- derived MP without affecting endothelial MP in post-myocardial infarction patients (Turco, et al., 2008). Thus there is for now no evidence that EPA and DHA, taken together or individually, may improve EPC, MP, CEC or CPC in humans.

NOx were consistent with data previously reported in healthy populations (McBrien, et al., 2012). Very few studies have examined the effect of fish oils on

plasma NOx, preferring the measurement of circulating markers of EF (such as E-selectin, VCAM-1, ICAM-1), or non invasive measurements like the FMD or the forearm blood flow, described in Chapter 1. Worth mentioning, dietary nitrate (from certain vegetables such as beetroot) contributes to the circulating pool of NO. Because our NO assay was an add-on to the project, we did not restrict dietary nitrate prior to the study, which may have influenced the results. In contrast with our findings, a 12 week diet enriched with mackerel-derived products, providing 2.5 g of EPA+DHA per day has been reported to increase plasma NOx levels in elderly (De Berrazueta, et al., 2009), and this was associated with an improvement of forearm blood flow in response to reactive hyperaemia, while other markers of endothelial activation such as von Willebrand factor and E-selectin remained unchanged. In this case, the authors did not report that they controlled for or restricted nitrate dietary intake.

In healthy subjects, there is also growing evidence that fish oils improve EF, as measured by non-invasive techniques, such as FMD or FBF (Chin, et al., 1993, Khan, et al., 2003, Shah, et al., 2007, Walser, et al., 2006). However none of the fish oil studies in healthy subjects have investigated the effect of fish oils on plasma NOx or EPC. In a design similar to ours (6 weeks, 4g/d), Mori *et al.* showed that DHA improved vasodilation in overweight mildly hyperlipidaemic subjects, as measured by FBF in response to acetylcholine (Ach) as well as sodium nitroprusside (SNP) or Ach+L-NMMA, suggesting that the vasodilatory mechanisms of DHA are endothelium-independent, while EPA had no effect (Mori, et al., 2000). In contrast, it was later shown that lower doses of EPA alone (1.8 g/d) provided for 12 weeks were able to improve FBF to ACh but not to SNP in hypertriglyceridemic subjects, suggesting EPA exert vasodilatory actions that are endothelium-dependent (Okumura, et al., 2002). More recently, corroborating our results, Egert *et al.* showed that there was no change of circulating markers of endothelial activation, including VCAM-1, ICAM1, E-selectin and endothelin-1 in healthy participants after 3 weeks of supplementation with either EPA nor DHA at 4 g/d, in a crossover design (unpublished results, see (Ueshima, et al., 2007) for reference). The individual variability, as well as the variability in design and supplement composition may account for the discrepancies. Longer interventions are needed to determine the differential effect of EPA and DHA on EF, as well as a standardisation of the techniques. FMD is the gold standard technique to assess EF for now, but has a high inter- and intra-observer variability (Lee, et al., 2002).

Fish oils have been shown to improve EF as measured by non invasive techniques (FMD, FBF), but their effect on circulating markers of EF remains uncertain, and data on the separate effect of EPA and DHA are limited. The development and standardisation of techniques assessing circulating markers of EF, including EPC but also circulating endothelial cells and endothelial microparticles, (Li, Tse et al. 2011), may help to further our understanding of the effects of EPA and/or DHA on EF.

3.3.3 Microvascular function (capillary density)

The capillary network plays a major role in distributing blood flow and pressure to tissues and organs (groom, 1996) and its rarefaction has been associated with several chronic diseases such as diabetes (Campbell and Hemmelgarn, 2012) and hypertension (Antonios, et al., 1999). Capillary rarefaction may be primary (precedes the onset of hypertension) or secondary (is a consequence of high BP) (Gee, et al., 2012). It has been suggested that capillary rarefaction occurs in 2 phases: functional, then anatomic (Prewitt, et al., 1982). Capillary rarefaction appears to be primarily functional in hypertension and overweight individuals (Czernichow, et al., 2010, Serné, et al., 2001), although an impairment in structural capillary density has also been observed in hypertensive (Antonios, et al., 1999, Clark, et al., 2012). Functional capillary density is observed at rest, while anatomic (structural) capillary density (Cheng, et al., 2008) is measured during the inflation of a cuff at low BP (usually 60 mmHg, 5 min), which induces ischaemia and recruits all capillaries present in the deeper layers of the epidermis to the surface (Antonios, et al., 1999, Campbell, 2012). Several drug interventions have aimed at improving capillary density, both functional and structural, especially in diabetes (Campbell and Hemmelgarn, 2012) and hypertension (Gee, et al., 2012). To our knowledge, nutritional interventions have only investigated the effect of salt intake on capillary density, showing that a modest reduction of salt intake (9.7 to 6.5g/d) for 12 weeks increased both functional and anatomic capillary density in subjects with untreated mildly raise BP (He, et al., 2010). In our study, functional capillary density, which has been associated with EF (Cheng, et al., 2008, Cheng, et al., 2008), remained unchanged upon EPA and DHA treatment. This contrasts with the few studies that investigated the effect of fish oil supplementation in animals reporting a beneficial effect on capillary density in the ventricles of hypertensive rats (Mitasikova, et al., 2008), and in the cheek pouch of hamsters, associated to an increased arteriolar blood flow (Conde, et al., 2007). In the latter, these beneficial effects were attributed to the ability of fish oils to modulate eicosanoid synthesis, Ca^{2+} or K^{+} channel activity, as

well as NO production, which can regulate vascular tone, thus influencing the blood flow in arterioles as well as capillary function and density (Conde, et al., 2007).

The EDT study is - to our knowledge - the first study looking at the effect of fish oils on capillary density in humans. Long chain *n*-3 PUFA, especially DHA have been associated with reduced BP (Liu, et al., 2011), which relates to microvascular function and capillary density (Serne, et al., 1999). In addition microvascular function relates to EF and NO production (Cheng, et al., 2008, Cheng, et al., 2008, Conde, et al., 2007), which may be improved by fish oils (Chin, et al., 1993, Khan, et al., 2003, Shah, et al., 2007, Walser, et al., 2006). In our study, EPA and DHA failed to affect NO production and EPC levels (see 3.3.1), as well as BP (see 3.3.5), which is in line with the lack of effect on capillary density.

3.3.4 Arterial stiffness and peripheral resistance

SI-DVP and RI-DVP, as well as the peripheral and central AIx, were consistent with data previously observed in healthy people (Evison, et al., 2006, Hall, et al., 2008, O'Rourke, et al., 2001) and remained unchanged upon EPA and DHA treatment. Conversely, a recent meta-analysis suggests that intervention with fish oils improve arterial stiffness (Pase, et al., 2011), however these results should be appropriately interpreted. Nine of the ten trials considered in this meta-analysis involved populations at risk, including overweight, diabetic, hypertensive, dyslipidaemic or hyperlipidaemic, while only one trial considered healthy young males. Importantly, the latter – whose data were unpublished - showed that fish oils (1.8g EPA + 1.1 g DHA / day) had no effect on radial-carotid PWV (See (Damsgaard, et al., 2008) for reference). Noteworthy, four of the ten trials investigated PWV (two at the brachial-ankle and two at the cardio-ankle and radial-carotid sites) and six investigated as capacity compliance and systemic compliance, but none of them looked at carotid-femoral PWV, central haemodynamic indices nor any other indices of arterial stiffness measured in our study. The effect of fish oils on carotid-femoral PWV was recently reported by Sanders *et al.* who showed that low doses of EPA and DHA (0.45-1.8g/d) for 12 months in healthy subjects (aged 45-70 y) had no effect (Sanders, et al., 2011). In fact, our findings are in accordance with the only study – to our knowledge - that investigated the impact of fish consumption on the augmentation index specifically. Hallund *et al.* recently reported that 8 weeks of trout consumption (150g / day), providing 2.0 and 0.9 g/d of DHA and EPA, respectively, had no effect on the augmentation index – as well as PWV – in

healthy males, compared to 150g of chicken per day (Hallund, et al., 2010). It should be noted that this study was not included in Pase's meta-analysis that only looked at fish oil supplements, but not dietary fish intervention. Together with our findings, this suggests that relatively high doses of fish oils (~2-3g/d) given for less than two months are not able to improve central and peripheral indices of arterial stiffness/vascular tone, in healthy subjects. Although a difference in the rate of arterial stiffening would not be expected over the duration of supplementation used in the current study, these indices of arterial stiffness are also influenced by peripheral resistance, and could therefore be influenced by shorter term changes in vasodilation. The unchanged levels of NOx and BP corroborate the absence of effect of EPA and DHA on PWA and DVP indices in our study.

3.3.5 BP and HR

Although the hypotensive role of fish oils is well-established in subjects at higher CVD risk, such as elderly or hypertensive patients (Geleijnse, et al., 2002, Mori, 2006), a lack of effect of fish oils on SBP and/or DBP is not uncommon in healthy people (Conquer and Holub, 1998, Damsgaard, et al., 2008, Morris, et al., 1993, Sanders, et al., 2006, Sanders, et al., 2011). Similarly, the HR lowering effect of fish oils is clear in subjects with higher baseline values (Mozaffarian, et al., 2005) but a lack of effect in healthy population is not unusual (Damsgaard, et al., 2008, Sanders, et al., 2011, Walser, et al., 2006). It is therefore not surprising that EPA and DHA both failed to lower seated and supine HR and BP and ambulatory BP in the EDT study. Although we did not observe an effect on ambulatory 24h and daytime HR, night time HR was decreased by 3 bpm by DHA and increased by 2.7 bpm upon EPA treatment. From the few studies investigating the individual effect of EPA and DHA on BP and HR, it appears that the HR and BP lowering effects of fish oils are due to DHA rather than EPA (Grimsgaard, et al., 1998, Mori, et al., 1999, Theobald, et al., 2007). Interestingly, Grimsgaard *et al.* showed that while having no effect on BP, DHA (4g/d, 7 weeks) lowered HR in healthy males by 2.2 bpm while EPA increased it by 1.9 bpm in healthy males (Grimsgaard, et al., 1998), which is consistent with our findings for HR at night. In mildly hyperlipidaemic males, Mori *et al.* showed that 24h, daytime and night time HR increased in the EPA group by 2.0 bpm, 2.7 bpm, and 2.3 bpm, respectively, without reaching significance, while they were all significantly decreased by DHA (4g/d, 6 weeks) (Mori, et al., 1999).

The mechanisms by which EPA and DHA could exert opposite effects on HR remain unclear. Both EPA and DHA were shown to modulate potassium, sodium and calcium channel activities in myocardial cells, regulating myocyte electrical excitability and contractility (Li, et al., 2009, Xiao, et al., 1997, Xiao, et al., 2005). These effects, observed in a concentration-dependent manner, are thought to be mediated by the effect of EPA and DHA on membrane fluidity (Xiao, et al., 2005), although other mechanisms, such as direct binding of *n*-3 LCP to the channel could be involved (Kang and Leaf, 1996). The cardioprotective effects of fish oils are believed to be due – at least partially – to their incorporation in cardiac membrane cells (McLennan, 2001, McLennan, et al., 2011), and there is growing evidence from animal studies that DHA, compared to EPA, is preferentially incorporated (McLennan, 2001). Collectively, these findings help to explain the anti-arrythmic and HR-lowering effects observed with DHA but not EPA in humans (Mori, et al., 1999). In addition, incorporation of DHA into the membrane of cardiomyocytes influences the beta adrenergic system to a greater extent than EPA (Grynberg, et al., 1995), potentially an important mechanism in the hypotensive and anti-arrhythmic effects of DHA. DHA incorporation into the membrane of endothelial cells stimulates ATP release from the endothelium, increasing vasodilation by stimulating nitric oxide (NO) release (Hashimoto, et al., 1999). The induction of NO release, together with the decrease in noradrenaline levels, are likely to be responsible for the BP-lowering effect of DHA (Hashimoto, et al., 1999). It should be noted that in contrast with observations in animal cardiac membranes (McLennan, 2001), EPA appears to be incorporated in humans to a greater extent than DHA, in various lipid fractions and cells (Katan, et al., 1997). Whether this is the case in human cardiac membranes remains uncertain as it is evidently complicated to observe in humans.

Whether the changes in HR in humans are due to changes in EPA, DPA or DHA in the cardiac membrane remains unknown. Erythrocyte composition has been shown to be the most reliable lipid fraction in blood to represent cardiac membrane composition (Campbell, et al., 2012). Interestingly, HR was strongly correlated with DPA at the end of intervention, but not with other VLC *n*-3 PUFA. Conversely, in Grimsgaard *et al.*'s study changes in HR were inversely correlated with changes in serum phospholipid DHA, and the authors observed a significant decrease in serum phospholipid DHA in the EPA group, where HR was increased (Grimsgaard, et al., 1998). Overall, whether the increase in HR sometimes observed with EPA is due to EPA itself, its conversion to

DPA, or DHA deprivation - as previously observed in plasma and platelet phospholipids upon EPA supplementation (Grimsgaard, et al., 1997, Mori, et al., 2003) - is yet to be elucidated.

3.3.6 Lipid profiles

3.3.6.1 Plasma fasting triacylglycerol and total NEFA concentrations

Neither EPA nor DHA affected plasma TAG concentrations compared to placebo. While 3g of fish oils per day have shown a hypotryglyceridemic effect, particularly in individual with higher baseline levels (Mozaffarian and Wu, 2011), a null effect is not rare in normolipidaemic humans (Harris, 1996, Osterud, et al., 1995). However, there was a small but significant NEFA-lowering effect of EPA and DHA, particularly following EPA supplementation. Although the TAG lowering effect of fish oils is well-established, it was previously reported that only half of placebo-controlled trials involving normotriglyceridemic subjects showed a significant effect of fish oils on TAG levels, vs. 75% in hypertriglyceridemic patients (≥ 2mM) (Harris, 1996). Nonetheless, within-group analysis showed a significant decrease of TAG in the EPA group (-26.8%) but not in the DHA or placebo group. A preferential effect has been observed in slightly hypertriglyceridaemic subjects where 3 weeks of supplementation with EPA or fish oil but not DHA alone decreased TAG levels (Rambjor, et al., 1996). However, as the authors pointed out, the DHA group was relatively small (n = 9) compared to the EPA and fish oil group (n = 25 and 35, respectively) and thus underpowered. In addition, EPA is incorporated into various lipid fractions at a faster rate than DHA (Katan, et al., 1997), and three weeks of intervention may have been insufficient for DHA to exert its TAG lowering effect. Interestingly, seal oil (340 mg EPA, 230 mg DPA, 450 mg DHA) but not fish oil (210 mg EPA, 30 mg DPA, 810 mg DHA) reduced plasma TAG in healthy volunteers after two weeks of supplementation, and the authors suggested that seal oil may be more efficient in improving lipid profiles due to its high DPA as well as EPA content (Khan, et al., 2012).

Changes in TAG levels were inversely correlated with EPA levels in erythrocytes, but not DHA, which is consistent with earlier findings showing that TAG changes correlated with platelet EPA but not DHA after 6 weeks of supplementation with fish oils (3g EPA+DHA / day) in hypertriglyceridemic men (Leigh-Firbank, et al., 2002). Concurrent with this, previous animal and *in vitro* studies suggested that EPA was likely to be the antilipidaemic agent, due to its ability to stimulate mitochondrial

FA oxidation in the liver (Frøyland, et al., 1997, Madsen, et al., 1999), and to its direct impact on TAG synthesis, VLDL assembly and secretion (Berge, et al., 1999, Willumsen, et al., 1993). EPA decreased NEFA levels compared to placebo, which could potentially reduce FA supply for hepatic TAG synthesis. However, reduced TAG levels may themselves be responsible for a reduction of NEFA release from the TAG-rich VLDL, in which case the effect observed on NEFA levels would be a consequence of reduced TAG rather than a cause (Harris and Bulchandani, 2006). Nonetheless, evidence from *in vitro* studies suggest that both EPA and DHA, as well as their derivatives, are able to regulate the expression of transcription factors in the liver, such as PPARs (Chambrier, et al., 2002, Itoh and Yamamoto, 2008) and SREBP-1 (Caputo, et al., 2010, Howell Iii, et al., 2009, Kajikawa, et al., 2009) involved in lipid metabolism, which may contribute to the regulation of TAG levels (Harris and Bulchandani, 2006). Consistent with this, supplementation with either EPA or DHA for at least 4 weeks have consistently shown that both EPA and DHA decrease TAG levels in healthy subjects (Egert, et al., 2009, Grimsgaard, et al., 1997, Olano-Martin, et al., 2010), as well as type 2 diabetics (Woodman, et al., 2002) and dyslipidaemics (Mori, et al., 2000, Nestel, et al., 2002), sometimes with a more potent effect of DHA (Buckley, et al., 2004, Grimsgaard, et al., 1997, Mori, et al., 2000). Whether the hypotriglyceridaemic effect of DHA usually observed in humans is due to a direct effect or partially to its retroconversion to EPA remains uncertain and the differential mechanisms by which of EPA and DHA may alter lipid metabolism remains to be fully elucidated.

3.3.6.2 Cholesterol

The unaffected lipoprotein levels, including total cholesterol levels, LDL or HDL upon EPA or DHA treatment are in accordance with the majority of previous studies in healthy people that have reported little or no effect of fish oils or oily fish on HDL and LDL (Hallund, et al., 2010, Kong, et al., 2007, Maa, et al., 2007). Recent meta-analyses, including patients at high CVD risk, show that although fish oils show a consistent hypotriglyceridemic effect, they have no effect on total cholesterol and only slightly – but significantly – raise LDL and HDL, suggesting that fish oils are unlikely to exert a cardioprotective effect through a role in cholesterol metabolism (Balk, et al., 2006, Eslick, et al., 2009). However, those analyses did not consider the total cholesterol/HDL ratio or the size of LDL particles, which were also reported as independent predictors of CV events (Mewshaw, et al., 2007, Rizzo and Berneis, 2006,

Ullrich, et al., 2007). Although there was no difference between groups, within group analysis showed a slight but significant decrease in the ApoB/LDL ratio (suggesting an increase in LDL size) and total cholesterol/HDL ratio in the EPA group, but not the DHA group. This contrasts with previous studies, generally showing an improvement in both LDL and HDL size upon fish oil supplementation, especially upon DHA treatment. In hyperlipidemic men, DHA increased LDL levels and size, as well as HDL size but not HDL levels, while EPA had no effect (Mori, et al., 2000). In healthy men (20-70 y old), within group analysis showed that DHA but not EPA increased HDL size (Buckley, et al., 2004), while there was no difference between groups. A beneficial effect on total cholesterol/HDL ratio has been observed after supplementation with fish oils (Harris, 2007) as well as EPA or DHA alone (Conquer and Holub, 1996, Grimsgaard, et al., 1997). Noteworthy, TAG/HDL ratio was significantly decreased within the EPA group (~ -30%, $P < 0.007$), while only a trend was observed in the DHA group (- ~21%, $P = 0.072$), and no change was observed with placebo. TAG/HDL ratio is believed to be a stronger predictor of CV events than the LDL/HDL ratio or TAG levels on its own (Wahamaa, et al., 2007), and our results may indicate a beneficial role of both EPA and DHA on lipid metabolism, despite the lack of significant effect of DHA on TAG levels.

3.3.7 Oxidative stress and platelet function

3.3.7.1 Isoprostanes

Because of the large array of substances the endothelium secretes, numerous impairments are associated with endothelial dysfunction, other than the one resulting from the vasodilation/vasoconstriction described in chapter 1. Oxidative stress, i.e. the imbalance between pro- and anti-oxidant activity, plays a pivotal role in endothelial dysfunction and the pathogenesis of CVD (Shah, et al., 2010). Oxidative stress involves the accumulation or superoxide anion in the mitochondria of endothelial cells, and VSMC. Reactive oxygen species injure the endothelial cell and nuclei, interact with eNOS cofactors and NO, contributing to the alteration of vasomotion (Wong, et al., 2010). ROS and NO are also responsible for lipid peroxidation and lead to the formation of oxidised LDL, which plays an important role in the development of atherogenesis (Steinberg, 1997). Oxidative stress may thus both precede and exacerbate endothelial dysfunction as well as the development of atherosclerosis. Several markers of oxidative stress, such as oxidised LDL or isoprostanes have emerged as valuable tools for the

diagnosis and prognosis of CVD (He, et al., 2010, Sotirios, 2006), and a therapeutic target (Sotirios, 2008).

Plasma isoprostanes concentrations were similar to those previously reported in healthy subjects (Levine, et al., 2001, Upritchard, et al., 2003) and remained unchanged upon both EPA and DHA treatment, which is in line with the lack of effect on EF. Our results are also consistent with previous findings in humans, where fish oils failed to reduce levels of diverse markers of oxidative stress, such as malondialdehyde or oxidised LDL (Filaire, et al., 2010, Hanwell, et al., 2009, McAnulty, et al., 2010), as well as urinary F2-isoprostanes (Petersson, et al., 2010, Ulven, et al., 2011). However there is inconsistency as others have shown that fish oils or oily fish reduced isoprostane levels in diabetics (Mori, et al., 1999, Mori, et al., 2000), overweight hyperlipidemic (Mori, et al., 2000), post-menopausal women (Higdon, et al., 2000) or healthy subjects, at doses as little as 200mg/d DHA (Guillot, et al., 2009). It should be noted, however that these studies have measured urinary excretion of isoprostanes, rather than plasma concentrations. In contrast to our results, Mas *et al.* reported that both EPA and DHA (4 g/d) reduced plasma F2-isoprostanes after 6 weeks of intervention in overweight, dyslipidaemic men as well as in treated-hypertensive Type 2 diabetic, patients (Mas, et al., 2010).

In vitro studies suggest that EPA and DHA may improve oxidative stress, for example by regulating the uptake of oxLDL by macrophages (McLaren, et al., 2011), or competing with AA in the activation of NADPH oxidase (Vignais, 2002) and in the formation of AA derived isoprostanes. However, with their high degree of unsaturation, EPA and DHA are particularly prone to oxidation and may contribute to the oxidative stress. In support of that isoprostanes of the F-3 series can be derived from EPA, and have been proposed as a valuable index of oxidative stress, particularly in people with high intake of *n*-3 PUFA (Chang, et al., 2008, Song, et al., 2009), and may be of interest in fish oil supplementation studies. This duality may explain that there is to date no clear evidence that fish oils may reduce oxidative stress and lipid peroxidation in humans, and further research is needed to determine whether fish oils may be beneficial.

3.3.7.2 PMA

Previous research investigating the effect of fish oils on platelet function has mainly focused on platelet aggregometry - measuring *ex vivo* platelet activation - and measurements of thromboxane synthesis, and there is uncertainty regarding the correlation between platelet activity *ex vivo* and *in vivo*. In general, fish oils seem to

reduce platelet aggregation and thromboxane A2 production in response to ADP and collagen in healthy people (Mann, et al., 1997, Siess, et al., 1980), type 2 diabetics (Hartweg, et al., 2009) and in subjects with mildly raised blood pressure and cholesterol levels (Mori, et al., 1997). The measurement of leukocyte-platelet aggregation, including platelet monocyte aggregation allows the evaluation of platelet function *in vivo*. Din *et al.* recently showed that 500g of mackerel per week (providing 1g EPA+DHA / day) for 4 weeks was sufficient to reduce platelet aggregation by 35% in healthy men, without affecting markers of platelet activation (sP-selectin, sCD40L) (Din, et al., 2008). This contrasts with the lack of effect of either EPA or DHA on PMA in our study. The difference may be due to the variability of technique or individual variability.Our population had PMA levels of ~9% at baseline, while they were reduced from ~24% to ~16% after the 4 weeks of fish oil supplementation in Din *et al.* study. All of these levels have been previously reported in healthy populations (Lukasik, et al., 2011, Tapp, et al., 2011). One explanation for the current findings might be that both EPA and DHA together are necessary to improve platelet aggregation. However, several studies have shown that either EPA (Nomura, et al., 2003) (Wojenski, et al., 1991) or DHA (Guillot, et al., 2009, von Schacky and Weber, 1985, Woodman, et al., 2003) alone could improve platelet aggregation, as observed by the more traditional techniques of aggregometry. Interestingly, DHA, but not EPA, improved platelet aggregation in response to collagen and PAF in type 2 diabetics (Woodman, et al., 2003).

Mechanisms by which fish oils may modulate platelet function have been mostly attributed to the ability of EPA to compete with arachidonic acid (AA) in the COX pathway, leading to the formation of eicosanoids from the 3-series, less pro-thrombotic than the 2-series eicosanoids derived from AA (Krämer, et al., 1996, Tapiero, et al., 2002). On the other hand, DHA may exert a greater effect on platelet membrane fluidity compared to EPA (Hashimoto, et al., 2006), probably because of its additional double bond (Hashimoto, et al., 1999). EPA and DHA may also exert their anti-thrombotic effect on the endothelium, by stimulating the production of EPA derived PGI_3 which adds on to the anti-aggregatory effect of PGI_2 (Hishinuma, et al., 1999), inhibiting platelet activating factor synthesis (Mayer, et al., 2002) or stimulating eNOS activity (Li, et al., 2007, Li, et al., 2007). The decrease in PAF levels, as well as the increase of NO, which has anti-aggregatory properties, may also contribute to the anti-thrombotic effects of fish oils (Serhan and Chiang, 2008).

Both EPA and DHA appear to reduce platelet aggregation in humans (Din, et al., 2008), and DHA may be more potent (Woodman, et al., 2003). The reason why we did not observe any change in platelet monocyte aggregation remains unclear, but may simply be due to individual variability.

3.3.8 Glycemia and insulin sensitivity

As expected, six weeks of supplementation with EPA or DHA did not affect plasma glucose, insulin and indexes of insulin sensitivity in healthy young males. This is consistent with recent meta-analysis showing that fish oils had no significant effect on insulin, glucose and glycated hemoglobin levels (Balk, et al., 2006, Hartweg, et al., 2008). More recently, Akinkuolie *et al*. were the first to publish a meta-analysis assessing the effect of fish oils on indexes of insulin sensitivity and included all populations. They concluded that fish oils had no effect on insulin sensitivity overall (11 trials, *n* 618), although subgroup analysis showed a significant improvement in the group where insulin sensitivity was measured by HOMA (Akinkuolie, et al., 2011). The authors could not investigate dose and time effect of the interventions due to the limited number of trials, but these factors, as well as individual variability, may contribute to the discrepancies observed in human interventions (Fedor and Kelley, 2009). In addition, lack of standardisation of the methods used to assess insulin resistance (Muniyappa, et al., 2008), and the differences in the FA composition of the capsules, may play a role in the variability of response to treatment. To date, only a few studies have compared the role of EPA and DHA on insulin sensitivity and glycaemic control and as for EPA+DHA taken together, results are inconsistent. Corroborating our findings, Egert *et al.* reported a lack of effect of both supplements (2.8-2.9 g/d, 6 weeks) in healthy participants on insulin levels and sensitivity (Egert, et al., 2008); however EPA tended to increase glucose levels. In treated hypertensive EPA or DHA (3.7-3.8 g/d, 6 weeks) did not affect insulin levels, secretion and sensitivity but both increased glucose concentrations (Woodman, et al., 2002). In contrast, EPA and DHA (4 g/d, 6 weeks) both decreased insulin levels in hyperlipidaemic subjects while EPA tended to increase fasting glucose (Mori, et al., 2000). Although there is inconsistency in humans, there is growing evidence from animal studies that both EPA and DHA exert an insulin-sensitising effect (Fedor and Kelley, 2009) through their anti-inflammatory effect (Calder, 2009, Caughey, et al., 1996, Endres, et al., 1989, Meydani, et al., 1991), t ability to modulate adipokine production by the adipose tissue (Arai, et al., 2009), as well as the expression of transcription factors such as SREBP-1c, PPAR α and γ,

involved in glucose and lipid metabolism (Caputo, et al., 2010, Chambrier, et al., 2002, Howell Iii, et al., 2009, Itoh and Yamamoto, 2008, Kajikawa, et al., 2009). This contrasts with the discrepancy observed in humans and may suggest that more long term interventions (6-12 months) are required to determine whether EPA and/or DHA can improve adipokine levels and insulin sensitivity in humans.

3.3.9 Conclusion and limitations

We showed that neither EPA nor DHA improved endothelial and microvascular function, as assessed by NOx, EPC and functional capillary density measurements, The low levels of EPC in mononuclear cells limits the reliability of the method used, and research is still needed to standardise EPC measurement. Populations at high CVD risk, as well as other aspects of microvascular function, such as red blood cell velocity or capillary structure, may be considered in future intervention studies with fish oils.

DHA decreased and EPA increased heart rate at night, which corroborates previous findings (Grimsgaard, et al., 1998, Mori, et al., 1999). The reasons for this difference remain uncertain and may involve changes in EPA, DHA and/or DPA in the cardiac membrane. Pure forms of EPA should be used with caution when supplementing subjects at high CVD risk, or for long periods.

EPA and DHA showed differential incorporation into the erythrocyte and NEFA fractions. The dynamic of incorporation and conversion between the 3 VLC *n*-3 PUFA (EPA, DPA, DHA) may vary from one lipid fraction to another. The investigation of this dynamic within different lipid fractions (e.g. TAG, LDL, HDL) as well as circulating cells (e.g. inflammatory cells, platelets) or tissues (e.g. adipose tissue) could help further the understanding of the differential mechanisms by which EPA and/or DHA exert their cardioprotective effect.

Chapter 4 Effect of fatty acid profiles on endothelial function in human dermal microvascular endothelial cell

4.1 Introduction

NEFA derived from triacylglycerol-rich lipoproteins, as well as adipose tissue TAGs, are implicated in the pathogenesis of diabetes and metabolic syndrome (Boden, 1997). Increased NEFA levels, as observed in the insulin resistant state, inhibit glucose uptake in muscle (Peterson and shulman 2002), and contribute to oxidative stress and inflammation (Itani 2002). Beyond NEFA levels, NEFA composition, which partially reflects dietary habits, may play a crucial role in the development of endothelial dysfunction and insulin resistance. In humans, a high proportion of SFA in NEFA is associated with an increased 10-year CVD risk (Skidmore, et al., 2010), while long chain n-3 PUFA (especially EPA and DHA) correlate with markers of endothelial dysfunction (Yli-Jama, et al., 2002) and are associated to a reduced CVD risk (Rhee, et al., 2008, Yli-Jama, et al., 2002).

Endothelial cells are directly exposed to circulating lipids, and endothelial dysfunction precedes the onset of atherosclerosis and diabetes. Several studies have investigated the individual role of FA on endothelial function *in vitro*. Both EPA and DHA were reported to increase eNOS activity (Li, et al., 2007, Li, et al., 2007) and expression (Xue, et al., 2006) and to reduce inflammation. In contrast palmitic acid appears to reduce eNOS activity and NO production, probably through inflammatory mechanisms (Kim, et al., 2005, Wang, et al., 2006). However, some studies suggest that EPA and DHA may have deleterious effect on endothelial function (Gousset-Dupont, et al., 2007) and the effect of oleic acid remains uncertain (Couloubaly, et al., 2007, Kim, et al., 2005). This inconsistency may be due to variability in study design, cell types, treatment duration, form and doses of fatty acids used. Most *in vitro* studies have investigated the individual role of fatty acids, often dissolved in DMSO. Conversely, NEFA circulate in blood bound to albumin, as a mixture of SFA, MUFA and PUFA representative of the diet.

The microvascular network represents the largest endothelial surface, which makes it more vulnerable to endothelial dysfunction. The microcirculation ensures the delivery of nutrients, oxygen and other substances such as insulin to tissues (Campbell, et al., 2012) and a tightly controlled pressure, maintaining peripheral resistance (See

Chapter 1). Microvascular insulin resistance and dysfunction, as observed in obesity, diabetes or hypertension, involve the skin, skeletal muscle, cardiac muscle, retina and kidneys (Ko, et al., 2010, Picchi, et al., 2010) and may thus play a pivotal role in co-morbidities of these diseases. Drug or nutritional interventions targeting an improvement of microvascular function could be of great importance; and the understanding of endothelial function at the microvascular level appears crucial, and would lie in the scope of the microvascular measurements described in the previous chapter.

4.1.1 Aim and Objectives

The aim of this chapter is to investigate the effect of FA profiles - typical of dietary patterns - on endothelial function in microvascular endothelial cells, and examine the modulation of their response by insulin and inflammatory conditions. The production of the two major vasodilators released by the endothelium, nitric oxide (NO) and prostacyclin (PGI_2), was examined.

4.1.2 Hypothesis

It was hypothesised that NO production/eNOS expression would be decreased and increased by profiles enriched in saturated fats and *n*-3 PUFA, respectively. A secondary hypothesis was that PGI_2 would be increased upon *n*-6 PUFA treatment, and decreased by *n*-3 PUFA.

4.2 Material and methods

4.2.1 List of equipment and consumables

- LEEC Research CO_2 Incubator (cat.no. GA3N, LEEC Ltd., Nottinghamshire, UK)
- Eppendorf centrifuge (cat.no. 5804R, Eppendorf UK Ltd., UK)
- Synergy™ HT Multi-Mode Microplate Reader Bio-Tek® (Bio Tek, Bedfordshire, UK)
- KC4.v3.4 software Bio-Tek® (Bio Tek, Bedfordshire, UK)
- Dri-block heater (Techne cat.no. DB-2A, Thelabwarehouse Ltd., London, UK)
- Bio-Rad® Model 200/2.0 Power Supply (Bio-Rad Laboratories Ltd., Hertfordshire, UK)
- Mini-PROTEAN 3 Cell and Mini Trans-Blot® module (Bio-Rad Laboratories Ltd., Herts. UK)
- V10-SDB and V20-SDB semi dry blotters (Scie-Plas Ltd., Cambridgeshire, UK)
- Luckham rotatest R100 shaker (cat.no. 454-129, Genetic Research Instrumentation Ltd., Essex, UK)
- G:Box Chemi imaging system (Syngene, Scientific Laboratory Supplies Ltd, Nottinghamshire, UK)
- Genesnap image acquisition software (Syngene, Scientific Laboratory Supplies Ltd, Nottinghamshire, UK)
- Agilent 6890 Gas Chromatograph (Agilent Technologies)
- F96 MicroWell™ Plates, polystyrene clear (Nunc cat.no. 167008, Thermo Fisher Scientific, UK)
- Nunc Multidishes Nunclon™Δ, 24 wells (Nunc cat.no. 142475, Thermo Fisher Scientific, UK)
- Sterile Acrodisc® Syringe Filters with 0.2 µm Supor® Membrane (Pall Corporation, VWR International Ltd., UK)
- Chromacol glass vials and inserts for gas chromatography (Esslab Ltd., Essex, UK)
- Short plates (cat.no. 165-3308, Bio-Rad Laboratories Ltd., Hertfordshire, UK)
- Spacer Plates With 1.5 mm Integrated Spacers (cat.no. 165-3312, Bio-Rad Laboratories Ltd., Hertfordshire, UK)
- Polyvinylidene fluoride (PVDF) transfer membranes (Immobilion®-P, cat.no. IPVH00010, Millipore UK Ltd, UK)
- Extra thick blot paper (cat.no. 170-3960, Bio-Rad Laboratories Ltd., Hertfordshire, UK)

4.2.2 List of reagents and chemicals

4.2.2.1 Cell culture and lysis

- Human dermal microvascular cells (HDMEC-c Endothelial Cells, cryopreserved, cat.no. C-12210, Promocell, UK)
- Endothelial Cell Growth Medium MV KIT (ECGM-MV, cat.no. C-22120, Promocell, UK)
- Newborn calf serum (cat.no. N4637, Sigma-Aldrich, Dorset, UK)
- Medium 199 (cat.no. M0650, Sigma-Aldrich, Dorset, UK)
- Fatty acid-free bovine serum albumin (FA-free BSA, cat.no. 85041C, Sigma-Aldrich, Dorset, UK)
- Sodium linoleate (cat.no. L8134, Sigma-Aldrich, Dorset, UK)
- Sodium oleate (cat.no. O7501, Sigma-Aldrich, Dorset, UK)
- Sodium arachidonate (cat.no. A8798, Sigma-Aldrich, Dorset, UK)
- Sodium stearate (cat.no. S3381, Sigma-Aldrich, Dorset, UK)
- Sodium palmitate (cat.no. P9767, Sigma-Aldrich, Dorset, UK)
- Sodium cis-4,7,10,13,16,19-Docosahexaenoate (DHA, cat.no. D8768, Sigma-Aldrich, Dorset, UK)
- Sodium cis-5,8,11,14,17-Eicosapentaenoate (EPA, cat.no. E6627, Sigma-Aldrich, Dorset, UK)
- Endothelial basal medium (EBM, MCDB 131, cat.no. U15-011, PAA Laboratories, Somerset, UK)
- Endothelial cell growth supplement (ECGS, cat.no. E2759, Sigma-Aldrich, Dorset, UK)
- Foetal calf serum (FCS, cat.no. F7524, Sigma-Aldrich, Dorset, UK)
- FA-free foetal bovine serum (Equitech Bio, inc., TX, USA)
- L-Glutamine with Penicillin/Streptomycin (L-Gln-Pen-Strep, cat.no. P11-013, PAA Laboratories Ltd., Somerset, UK)
- Hydrocortisone (cat.no. H-0888, Sigma-Aldrich, Dorset, UK)
- Trypsin EDTA (1:250) (10x) (cat.no. L11-003, PAA Laboratories, Somerset, UK)
- Dulbecco's Phosphate Buffered Saline (cat.no. D-1408, Sigma-Aldrich, Dorset, UK)
- Gelatin from bovine skin (cat.no. G-9382, Sigma-Aldrich, Dorset, UK)
- Tumor necrosis factor α (TNF α, cat.no. 210-TA-010, R&D Systems Europe Ltd, UK)
- Insulin (Incelligent SG®, cat.no. 4502-01, Millipore UK Ltd, UK)

- Dulbecco's Phosphate Buffered Saline (cat.no. D1408, Sigma-Aldrich, Dorset, UK)
- Human dermal microvascular endothelial cells, cryopreserved, (HDMEC-c, cat.no. C-12210, Promocell, UK)
- Endothelial Cell Growth Medium-MV KIT (ECGM-MV, cat.no. C-22120, Promocell, UK)
- Endothelial basal medium (EBM) MCDB 131 (cat.no. U15-011, PAA Laboratories, Somerset, UK)
- Endothelial cell growth supplement (ECGS, cat.no. E2759, Sigma-Aldrich, Dorset, UK)
- Foetal calf serum (FCS, cat.no. F7524, Sigma-Aldrich, Dorset, UK)
- FA-free foetal bovine serum (Equitech Bio, inc., TX, USA)
- L-Glutamine with Penicillin/Streptomycin (L-Gln-Pen-Strep, cat.no. P11-013, PAA Laboratories Ltd., Somerset, UK)
- Hydrocortisone (cat.no. H0888, Sigma-Aldrich, Dorset, UK)
- Trypsin EDTA (1:250) (10x) (cat.no. L11-003, PAA Laboratories, Somerset, UK)
- Gelatin from bovine skin (cat.no. G9382, Sigma-Aldrich, Dorset, UK)
- sodium dodecyl sulphate (SDS, BDH AnalaR, VWR International Ltd., UK)
- tris(hydroxymethyl)aminomethane (Tris base, BDH AnalaR, VWR International Ltd., UK)

4.2.2.2 FA transesterification

- Toluene (BDH AnalaR, VWR International Ltd., UK)
- Methanol (HPLC grade, Fisher Scientific, UK)
- Acetyl chloride (Sigma-Aldrich, Dorset, UK)
- Potassium carbonate (K_2CO_3, BDH AnalaR, VWR International Ltd., UK) 6 % (w/v) in UltraPure water
- Hexane (HPLC grade, Fisher Scientific, UK)
- Pentadecanoic acid (C15:0, internal standard for NEFA,50 mg/mL in CHCl3:MetOH 2:1 v/v) (~99% by capillary GC, Sigma-Aldrich, cat no. P6125-5G, Dorset, UK)

4.2.2.3 Western Blot

- Ammonium persulfate (APS, cat.no. A3678, Sigma-Aldrich, Dorset, UK)
- N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma cat.no. T9281, Sigma-Aldrich, Dorset, UK)

- 30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1 ratio) (Protogel®, cat.no. A2-0072, National Diagnostics, Geneflow Ltd, Staffordshire, UK)
- SDS (BDH AnalaR, VWR International Ltd., UK)
- Tris base (BDH AnalaR, VWR International Ltd., UK)
- Bromophenol blue (BPB, cat.no. B8026, Sigma-Aldrich, Dorset, UK)
- 2-Mercaptoethanol (cat.no. M6250, Sigma-Aldrich, Dorset, UK)
- PageRuler™ Prestained Protein Ladder (Fermentas SM0671, Thermo Fisher Scientific, UK)
- Glycine (cat.no. G8898, Sigma-Aldrich, Dorset, UK)
- Marvel Original Dried Skimmed Milk Powder (Premier Food)
- TWEEN® 20 (cat.no. P5927, Sigma-Aldrich, Dorset, UK)
- Albumin from bovine serum (BSA, cat.no. A9647, Sigma-Aldrich, Dorset, UK)
- Coomassie Brilliant Blue G (cat.no. 6104-58-1, Acros Organics Thermo Fisher Scientific, UK)
- Acetic acid (BDH AnalaR, VWR International Ltd., UK)
- Sodium Azide (NaN_3, BDH AnalaR, VWR International Ltd., UK)
- Rat α-tubulin monoclonal antibody, clone YL1/2 (Chemicon cat.no. MAB1864, Millipore UK Ltd, UK)
- Rabbit eNOS polyclonal antibody (Cat.no., sc-67003 Santa Cruz Biotechnology®, inc., Insight Biotechnology Ltd, UK)
- Mouse COX-2 (29) monoclonal antibody (Cat.No. sc-19999, Santa Cruz Biotechnology®, inc., Insight Biotechnology Ltd, UK)
- Goat anti-rat Immunoglobulin G- Horseradish peroxidase (IgG-HRP) (cat.no. sc-2006, Santa Cruz Biotechnology®, inc., Insight Biotechnology Ltd, UK)
- Goat anti-rabbit IgG-HRP (cat.no. sc-2004, Santa Cruz Biotechnology®, inc., Insight Biotechnology Ltd, UK)
- Goat anti-mouse IgG-HRP (cat.no. sc-2005, Santa Cruz Biotechnology®, inc., Insight Biotechnology Ltd, UK)
- Pierce® enhanced chemilumescent (ECL) Western Blotting Substrate (cat.no. 32106, Fisher Scientific UK Ltd, UK)

4.2.2.4 Assay kits

- MTT cell proliferation assay kit (Cayman Chemical Europe, cat.no. 10009365, Axxora, UK)

- LDH Cytotoxicity assay kit (Cayman Chemical Europe, cat.no. 10008882, Axxora, UK)
- Endotoxin assay kit (E-toxate®, cat.no. ET-0200, Sigma-Aldrich, Dorset, UK)
- Nitrate/nitrite fluorometric assay kit (Cayman Chemical Europe, Cat. No. 780051, Axxora, UK)
- Pierce® BCA Protein Assay Kit (Thermo Scientific, Cat. No. 23227)
- 6-keto Prostaglandin $F_{1\alpha}$ EIA Kit (Cayman Chemical Europe, Cat. No. 515211, Axxora, UK)

4.2.3 Solutions and buffers

4.2.3.1 Cell culture and lysis

- Insulin was dissolved in HCl 10 mM at 10 mg/mL and sterile filtered (0.2 μm). Then it was diluted in sterile PBS at 100 μg/mL and stored at -20 ˚C. TNFα was diluted in sterile PBS-BSA 0.1 % at 10 μg/mL and stored at
- SDS buffer (2% SDS, 10% Glycerol, 50mM Tris pH 6.8)

4.2.3.2 Western blot

- *SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)*

- Lower gel (resolving gel): Tris-SDS Buffer pH 8.8 (375mM Tris, 0.1% SDS), 10% acrylamide, 0.4% APS, 0.08% TEMED
- Upper gel (stacking gel): Tris-SDS Buffer pH 6.8 (125mM Tris, 0.1% SDS), 4% acrylamide, 0.5% APS, 0.06% TEMED
- Loading buffer: 0.2 % BPB/2-Mercaptoethanol (4:1, v/v)
- Tank buffer: Tris-glycine-SDS PAGE buffer (10X) diluted in Ultra Pure water. (0.25M Tris, 1.92M glycine, 1% SDS) (National Diagnostics, Geneflow Ltd, Staffordshire, UK)

- *Transfer:*

- Transfer Buffer (192mM Glycine, 25mM Tris base, 20% methanol)

- *Protein detection*

- Washing buffer: PBS-tween 0.1%
- Blocking solution: 5% skimmed milk, PBS-tween 0.1%
- Gel staining solution: Coomassie brilliant blue 0.50g/L, 50% methanol, 10% acetic acid

- Gel destain solution: 5% methanol, 7% acetic acid
- Primary antibody solutions (stored at 4°C):
    - Rat α-tubulin monoclonal antibody: 1:5000 in PBS-tween 0.1%, 3% BSA, sodium azide
    - Rabbit eNOS polyclonal antibody: 1:1000 in PBS-tween 0.1%, 3% BSA, sodium azide
    - Mouse COX-2 monoclonal antibody 1:100 in PBS/0.1% Tween, 3% BSA, sodium azide
- Horseradish peroxidase (HRP) secondary antibody solutions (prepared on the day)
    - anti-rat antibody (for α-tubulin): 1:5000 in PBS/0.1%Tween, 3% skimmed milk
    - anti-rabbit (for eNOS): 1:2000 in PBS/0.1% Tween, 3% skimmed milk
    - anti-mouse (for COX-2):

4.2.4 Overview

Human dermal microvascular cells (HDMEC) were incubated with 4 different FA profiles, corresponding to NEFA levels typically observed in blood following different dietary patterns: high SFA, high MUFA, high n-3 PUFA and high n-6 PUFA. They were prepared at 2 different levels, 400 and 1000 μM, characteristic of healthy and type 2 diabetic levels, respectively. HDMEC were also incubated with a FA-free BSA medium (control), making 9 profiles tested in total. This was performed in the presence or absence of tumour necrosis factor α (TNFα) for the whole period of incubation, to mimic the chronic inflammation characteristic of insulin-resistant states. The cells were also stimulated with insulin for a shorter period to simulate the acute effect of insulin postprandially. NO and PGI2 production was measured in culture media for all conditions. In addition, eNOS and COX-2 protein expression was measured in cell lysates on HDMEC cultured with all profiles in absence of insulin or TNFα.

As outlined in Figure 4-1, 3 preliminary steps were necessary in order to assess the effect of the 8 FA profiles on EF in HDMEC:

1) *Preparation of the FA profiles*:

FA sodium salts were combined with bovine serum albumin (BSA), in order to provide the endothelial cells with FA in the same form as NEFA circulate in blood. Then the different FA-BSA complexes were combined to reach the desired levels in

profiles.

2) *Testing of the media to apply to the cells*

Endotoxins, mainly lipopolysaccharides, may be present in BSA or FA salts and may affect endothelial function, including NO production and prostacyclin production (Lu, et al., 1996, Myers, et al., 1992, Salvemini, et al., 1989, Schildknecht, et al., 2005). Thus, their concentration was measured in the media prior to the experiments. In addition, the viability of the cells upon FA profiles application must be estimated as well.

3) *TNFα and insulin levels determination*

Most of *in vitro* studies used high levels of insulin or TNFα, generally from 100 to 1000 times higher than physiological levels (Artwohl, et al., 2007, Eid, et al., 2007). Thus, different ranges of TNFα and insulin, from physiological levels to *in vitro* levels were used on a BSA (control) incubation, in order to see to what extent the treatment concentrations could be reduced to physiological levels while still exerting differential effects.

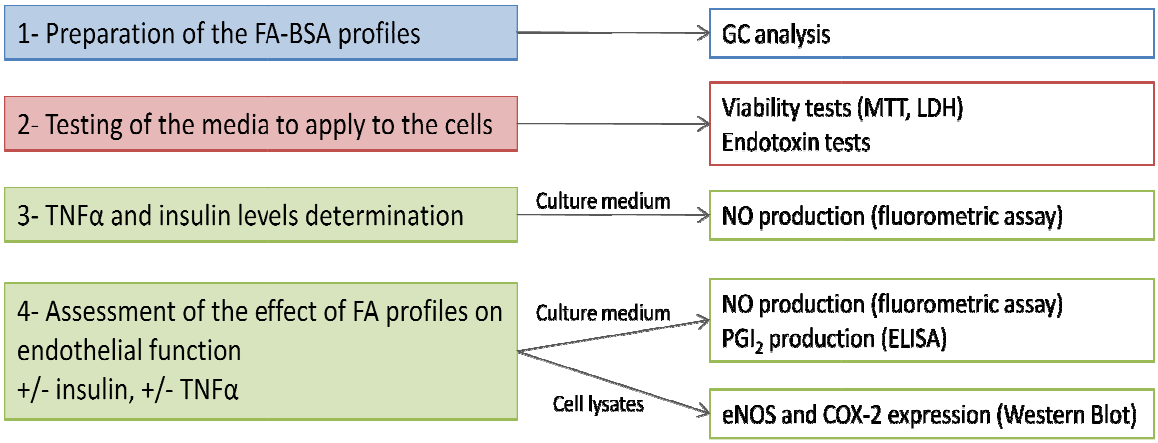

Figure 4-1: Outline of cell culture work

FA, fatty acids; BSA, bovine serum albumin; GC, gas chromatography; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; TNFα, tumor necrosis factor α; NO, nitric oxide; PGI_2, prostacyclin; ELISA, enzyme-linked immunosorbent assay, eNOS, endothelial nitric oxide synthase; COX-2, cyclooxygenase-2.

4.2.5 BSA mixture preparation

FA sodium salts were complexed to FA-free BSA as previously described (Shaw, et al., 2007). BSA was dissolved in endothelial basal medium for 30 min at 37 ºC at 2.4 mM (control) or to reach a final concentration of 2.4 mM after combination with FA (profiles). Palmitate (C16:0), stearate (C18:0), oleate (C18:1(n-9)), linoleate (C18:2(n-6)), arachidonate (C20:4(n-6)), eicosapentaenoate (C20:5(n-3)), docosahexaenoate (C22:6(n-3)) were dissolved at 50 mg/mL in water 30 min at 40 ºC for unsaturated fats, or 70 ºC for SFA (until a clear solution is formed). They were immediately added to the BSA/EBM solution to reach a final ratio FA:BSA of 6 mM:2.4mM and mixed thoroughly. Palmitate and stearate solutions needed to be sonicated for 30 min at 37 ºC to further dissolve particles and ensure solubilisation. The FA content of individual FA-BSA complexes was first measured by gas chromatography (GC), in order to take the yield of the FA-BSA complexation into account for the preparation of the final profiles (Appendix XXXIII).

FA-BSA complexes were blown over with nitrogen to prevent oxidation and stored at 4 ºC overnight until final mixing. On the next day, the FA mixtures were combined to form four FA profiles: SFA, MUFA, n-6 PUFA and n-3 PUFA (Table 4-1). The remaining individual FA-BSA solutions were stored at -20ºC for endotoxin assessment. The final mixtures were then sterile filtered (0.2 μm), aliquoted and stored at -20 °C.

Table 4-1: Expected composition of the BSA-FA mixtures in molar % of total FA

	SFA profile	MUFA profile	n-6 PUFA profile	n-3 PUFA profile
SFA	**55**	35	35	35
16:0 (PA)	37	23	23	23
18:0 (SA)	18	12	12	12
MUFA	30	**50**	35	35
18:1 (n-9) (OA)	30	50	35	35
PUFA	15	15	**30**	**30**
C18:2(n-6) (LA)	12	12	25	12
C20:4(n-6) (AA)	1	1	3	1
C20:5(n-3) (EPA)	1	1	1	8
C22:6(n-3) (DHA)	1	1	1	9
Total n-6 PUFA	13	13	**28**	13
Total n-3 PUFA	2	2	2	**17**

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid; EPA, eicospentadecanoic acid; DHA, docosahexaenoic acid

The profiles were thawed on the day of experiment and applied to the cells at a total FA concentration of 400 and 1000 μM, corresponding to the fasting plasma levels found in healthy and insulin resistant states, respectively. As BSA has been shown to stimulate cell growth, 2 BSA controls were first prepared (BSA 'high' and BSA 'normal'), i.e. one for each level of FAs. Following initial experiments, it was decided to use only one BSA control: prior to each experiment, BSA control solution was added in the 'normal FA' profiles to keep the same BSA level in all mixtures. Then the eight profiles were diluted in EBM, Pen-Strep-Gln (2 mM-100 units/ml-0.1 mg/ml) and delipidised foetal calf serum 2% The average level of BSA in the eight profiles was used as a control.

4.2.6 General cell culture conditions

HDMEC were cultured in an atmosphere of 95% air and 5% CO_2, in gelatine coated T75 flasks, in endothelial cell growth medium for microvascular cells (ECGM-MV), as recommended by the manufacturer. They were passaged 3 times in ECGM-MV before being frozen at -80 ºC in DMSO/NCS/ECGM v/v/v 1/4/5, 1 million cells/ml. For each experiment, HDMEC (P3) were defrosted, grown, fed and split once in ECGM-MV. HDMEC P4 were then grown and fed to 90% confluence in ECGM-MV. Then the medium was changed to endothelial basal medium endothelial basal medium (EBM), foetal calf serum 20%, endothelial growth factor 5 µg/mL, L-Gln-Pen-Strep (2 mM-100 units/ml-0.1 mg/ml), and hydrocortisone 0.2 ng/mL. This medium is a growing medium, which contains lower levels of growth factors than the ECGM-MV, and allows the cells to slowly adapt to resting conditions. Cells (P5) were seeded at a density of 50,000 cells/well in 24 well plates (500 µL final volume of EBM-FCS20%) and incubated in a 5 % CO_2 incubator at 37 ˚C for 24h. Then the cells were starved overnight in EBM - FCS 2% + Pen-Strep-Gln. This medium is a "resting" medium and allows the cells to adapt to their experimental environment containing EBM - delipidised FCS 2%. For all tests (viability tests, EF assessment), cells were submitted to the same preparation (outlined in Figure 4-2) and treated in triplicate.

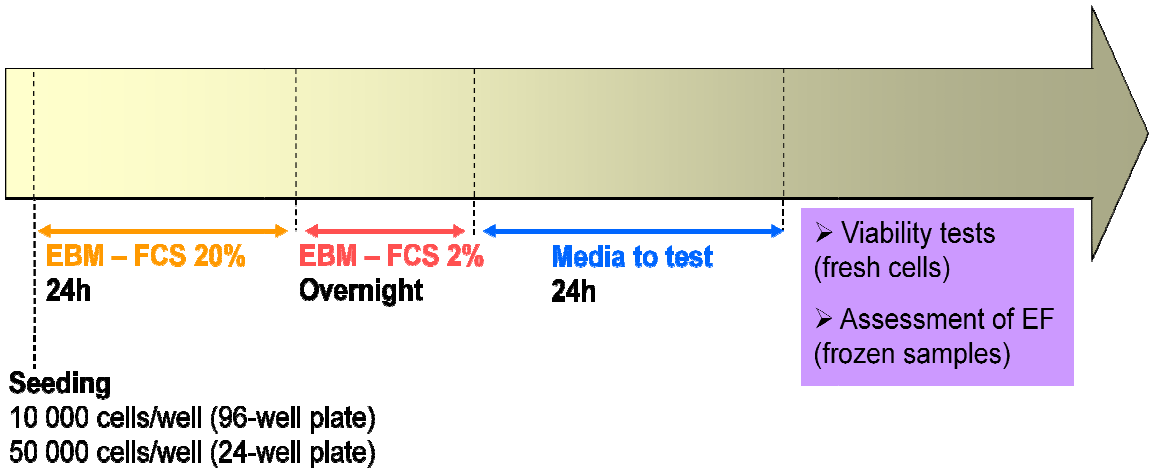

Figure 4-2: Treatment of HDMEC prior to viability tests, and endothelial function (EF) assessment.

EBM, endothelial basal medium; FCS, fetal calf serum; EF, endothelial function

4.2.7 Testing of the FA profiles

Before applying the testing media to HDMEC, the FA profiles prepared as described in Chapter 2 were tested for endotoxin content, cytotoxicity and their FA composition was checked.

4.2.7.1 FA composition

FA composition of the 8 FA profiles was tested after FA-BSA complexation, storage at -20ºC, and combination that was necessary prior to each experiment (described in Chapter 2). FA-BSA complexes were transesterified as previously described (Lepage and Roy, 1988) for gas chromatography (GC) analysis. The mixtures were briefly warmed up at 37˚C to get homogenous concentrations. 100µL of each FA profile were mixed with 100µL of internal standard (C15:0) 1mg/mL in methanol, and 2mL of methanol/toluene 4:1 (v/v). The tube was vortexed and chilled on ice while 200µL acetyl chloride were added drop-wise to avoid projections of the HCl produced by exothermic reaction. The resultant was incubated 2h at 60˚C and then neutralized with 5mL K_2CO_3 6%. After centrifugation at 1500g, 4˚C for 10min, the supernatants were transferred to vials for GC analysis.

GC conditions: The injection volume was 2 µL, the temperature was 160ºC for 4 min and then rose to 200ºC in 10 min (gradient of 12ºC/min).

4.2.7.2 Detection of endotoxin

Endotoxins (ETX) may be present even in sterile media and may affect the activity of the endothelial cells, particularly NO or PGI_2 production. Thus, the ETX content of each FA-BSA mixture that was used to make up the profiles was measured as described by the manufacturerAppendix . ETX standard was diluted in ETX free water to reach a range of 0.015-0.5 EU/mL. 100 µL of either sample or standard or ETX free water only (negative control) + 100 µL of working solution were transferred to bijou tubes and incubated for 1 h at 37 ˚C, undisturbed. Then the bijou tubes were inverted and the formation of a hard gel was considered as a positive result. All the profiles were tested for qualitative analysis, and then dilutions of BSA control were used for semi quantitative analysis. The lowest concentration found positive in ETX standard corresponds to the limit of detection of the test. For examples, if the lowest concentration of ETX standard found positive is 0.125 EU/mL, all samples found negative will have an ETX level inferior 0.125 EU/mL, corresponding to 1.25 ng/mL.

4.2.8 Viability tests

HDMEC (P5) were seeded at 10000 cells in 96-well plates in EBM – FCS 20% and incubated in a 5 % CO_2 incubator at 37 °C for 24h. Then the cells were starved overnight in EBM - FCS 2% and the 9 media to test were applied in triplicate for 24h (see Figure 4-3). The concentrated FA-BSA mixtures (prepared as described in 0) were diluted freshly on the day in EBM-delipidised FCS 2% + Pen-Strep-Gln to reach a final total FA concentration of 400 µM (Normal; N) and 1000 µM (High; H).

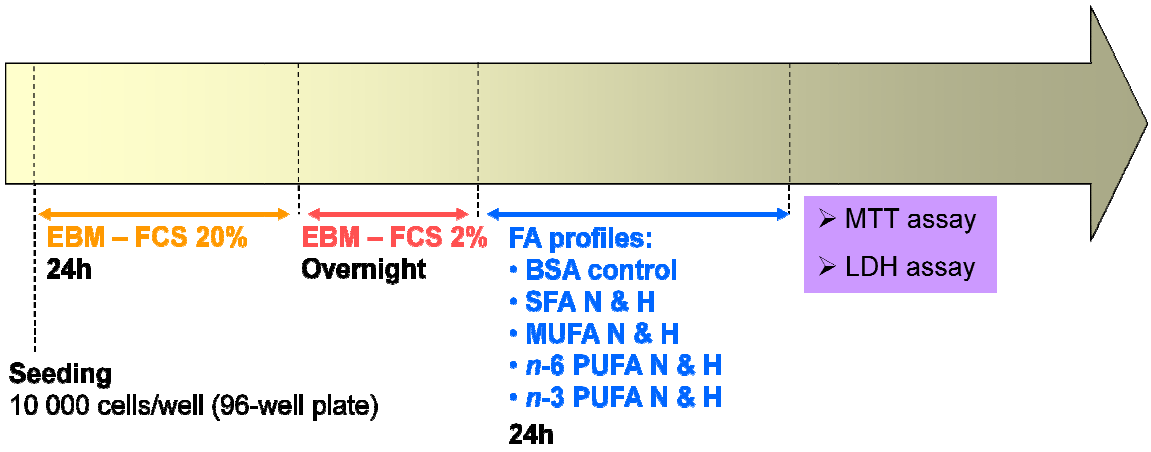

Figure 4-3: Treatment of HDMEC for viability tests

EBM, endothelial basal medium; FCS, fetal calf serum; FA, fatty acids; SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; N, normal levels (400 µM); H, high levels (1000 µM); MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.

4.2.8.1 MTT cell proliferation assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test is based on the ability of viable cells to uptake the MTT through the membrane and reduce it to formazan by intra-cellular NADPH oxidoreductase. The crystals of formazan are then dissolved with a dissolving solution, which gives a bright purple colour, absorbing around 550-570 nm. Thus, the absorbance is directly proportional to the amount of **viable** cells in the medium. Cells were seeded in triplicate following the treatment described in Figure 4-3 and the test was performed 6 times.as described by the manufacturer. Briefly, cells were incubated at 37 ºC in a 96 well plate for 24 h with the test media (100 µL/well), and 10 µL of MTT 5 mg/mL were added per well. The plate was incubated 4 h at 37 ºC and the medium was replaced by 50 µL dimethyl sulfoxide (DMSO). The plate was shaken 30 min at room temperature and read at 550 nm.

4.2.8.2 LDH Cytotoxicity assay

In addition to the MTT cell proliferation assay, an LDH (lactate dehydrogenase) cytotoxicity assay was performed twice. The LDH test is based on the characteristic of both apoptotic and necrotic cells that release LDH in the culture medium. LDH then oxidises the lactate into pyruvate, converting NAD^+ to $NADH,H^+$ at the same time. $NADH,H^+$ is then used as cofactor in the conversion of tetrazolium salts to formazan, catalysed by diaphorase. Unlike in MTT test, the formazan present in the medium gives an absorbance that is correlated to the amount of **non viable** cells. Cells were seeded in triplicate following the treatment described in Figure 4-3 and the assay was performed following the manufacturer's instructions. Briefly, cells were incubated at 37 ºC in a 96 well plate for 24 h with the media to test (120 µL/well). Then the plate was centrifuged 5 min, 400 *g*. 100 µL of either supernatant or LDH standard was then transferred to a new plate and 100 µL of reaction mixture were added per well. The plate was shaken 30 min at room temperature and read at 490 nm.

4.2.9 TNFα and insulin optimisation experiments

4.2.9.1 Preparation of reagents

TNFα and insulin solutions - stored at -20 ˚C - were defrosted and diluted in sterile PBS prior to each experiment, and 5 or 3 µL of the appropriate dilution of TNFα, insulin or PBS only (as a control) were added the 500 or 300 µL of culture medium, respectively.

As for viability tests, the concentrated FA-BSA mixtures (prepared as described in 4.2.5) were diluted freshly on the day in EBM-delipidised FCS 2% to reach the desired final concentrations. However, in order to keep the BSA levels comparable in all the profiles, BSA was added to the 'normal' FA profiles to reach the BSA levels in the 'high' profiles.

4.2.9.2 Cell treatment

HDMEC (P5) were seeded at 50000 cells per well in 24-well plates in EBM – FCS 20% and incubated in a 5 % CO_2 incubator at 37 ˚C for 24h. Then the cells were starved overnight in EBM - FCS 2% and were incubated for another 24 h with BSA control (final volume: 500 µL) (Figure 4-4). TNFα was applied for the whole length of incubation in order to mimic chronic inflammation as observed in IR state, while insulin was tested for a shorter period to simulate the postprandial acute effect observed physiologically. Time of incubation with insulin had to be defined as well, balancing

between its physiological acute effect and a longer period necessary for accumulation of NO or PGI_2 in the medium *in vitro*. Concentrations varied from physiological levels to higher levels commonly used *in vitro*. The concentration used for TNFα ranged from 0 to 10 ng/mL (24h), while insulin concentrations varied from 0 to 100 ng/mL (2 and 4h) (see Table 4-2). Culture media were collected in the end of the 24h incubation and stored at -80˚C for NOx measurements, cells were lysed with sodium dodecyl sulphate (SDS) buffer and cell lysates were stored at -20 ˚C for protein quantification by bicinchoninic acid (BCA) assay. The cells were treated in triplicate and the experiment was repeated 3 times.

Table 4-2: Concentrations of TNFα and insulin used in HDMEC culture in the presence of BSA control medium

TNFα (ng/mL), 24h		Insulin (ng/mL), 2h or 4h	
0.002	Physiological levels (healthy)	1	
0.01	Physiological levels (IR)	2	Physiological levels (PP)
0.02		5	
0.1		10	
1	*In vitro* levels	50	*In vitro* levels
10		100	

IR, insulin resistant state; PP, postprandial state

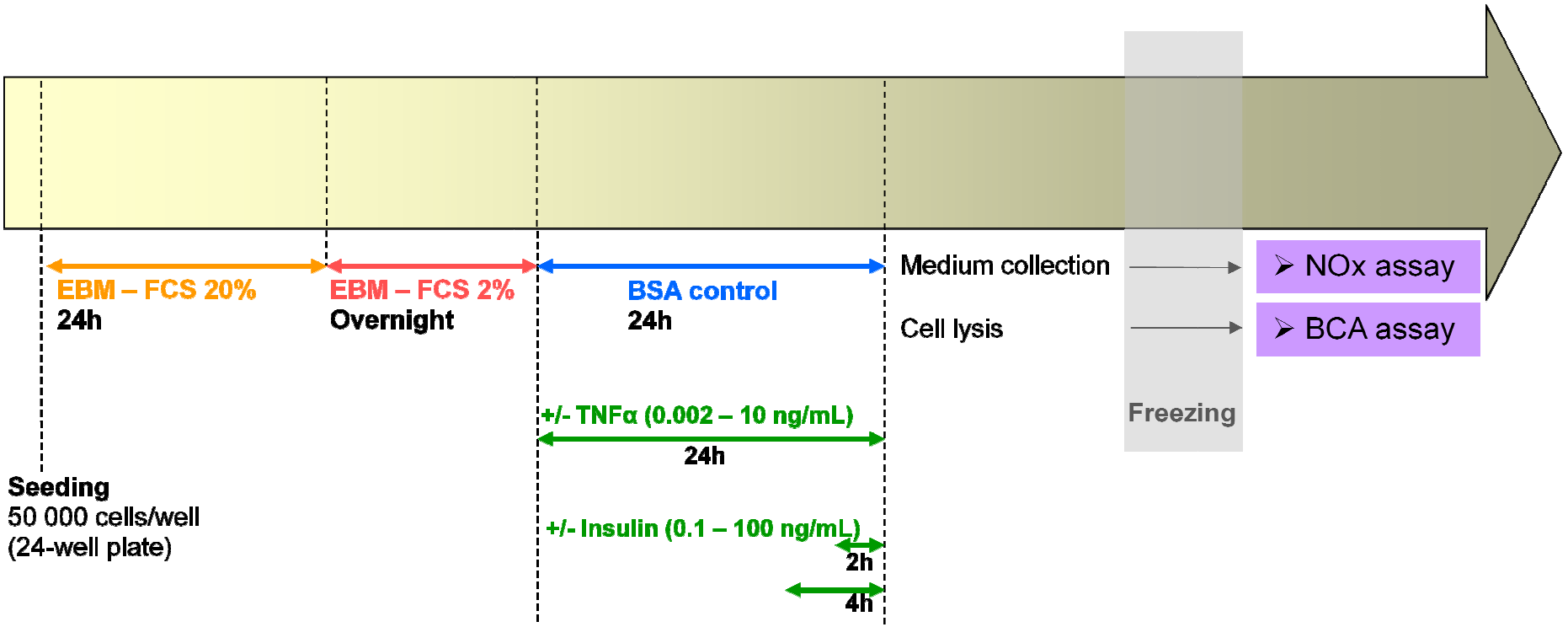

Figure 4-4: Treatment of HDMEC for determination of TNFα and insulin levels to use

EBM, endothelial basal medium; FCS, fetal calf serum; NOx, nitric oxide metabolite (nitrites / nitrates); BCA, bicinchoninic acid

4.2.10 Effect of FA profiles on endothelial function

HDMEC (P5) were seeded at 50000 cells per well in 24-well plates in EBM – FCS 20% and incubated in a 5 % CO_2 incubator at 37 ˚C for 24h. Then the cells were starved overnight in EBM - FCS 2% and were incubated for another 24 h with either BSA control, or the eight FA profiles previously described, in the presence of TNFα (24h) and/or insulin (2h) and/or PBS only (control). In order to increase NO concentration in the medium, the final volume of medium was reduced to 300 µL, and 3 µL of TNFα (24h) and/or insulin (2h) were added to reach the final concentrations of 10 ng/mL and 1 µg/mL, respectively. Culture media were stored at -80˚C for NO and PGI_2 analysis, cells were lysed with sodium dodecyl sulphate (SDS) buffer and cell lysates were stored at -20 ˚C for protein quantification by bicinchoninic acid (BCA) assay and Western blot analysis. The cells were treated in triplicate and the experiment was repeated 4 times.

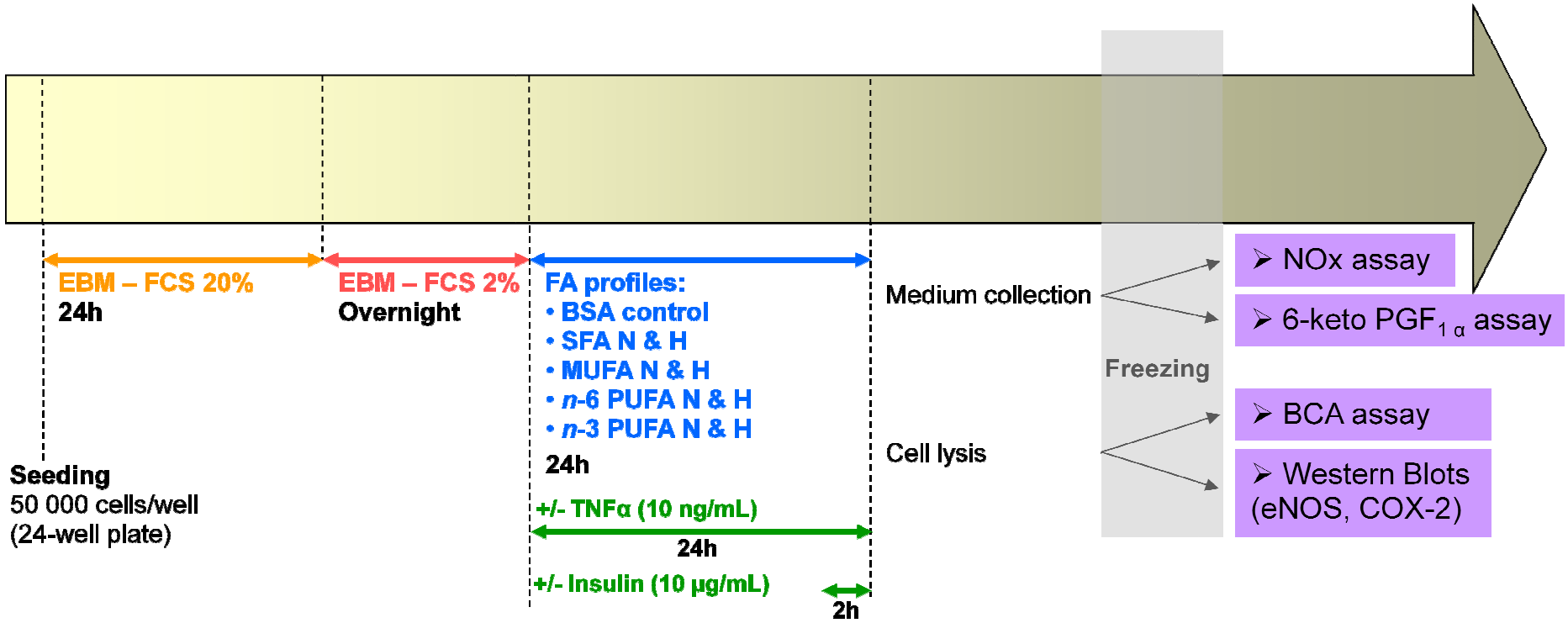

Figure 4-5: Treatment of HDMEC for viability tests

EBM, endothelial basal medium; FCS, fetal calf serum; FA, fatty acids; SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; N, normal levels (400 µM); H, high levels (1000 µM); NOx, nitric oxide metabolites (nitrites/nitrates); BCA, bicinchoninic acid (protein measurement); 6-keto PGF1$_{1\alpha}$, 6-keto prostaglandin F$_{1\alpha}$; eNOS, endothelial nitric oxide synthase; COX-2, cyclooxygenase-2.

4.2.11 BCA assay for total protein quantification

A BCA assay was performed in order to standardize NO and PGI_2 measurements, as well as eNOS and COX-2 protein expression, to the amount of proteins in the well, which is proportional to the amount of cells. BCA is based on the ability of proteins to chelate copper (Cu), leading to the reduction of Cu^{2+} to Cu^{+}. The latter is then chelated by the BCA to form a complex that strongly absorbs at 562 nm, proportionally to the amount of peptidic bonds. The test was performed following the manufacturer's instructions, with some modification in order to suppress the potential effect of SDS buffer - in which the samples are dissolved – on the absorbance. BSA standards were diluted in Ultrapure water. Samples were defrosted and heated 5 min at 95 °C in a heating block. 5 µL of either sample (cell lysate) or standard (0.0625-2mg/mL) were transferred per well, with 5 µL of either Ultrapure water or SDS buffer, respectively. Then 200 µL of working reagent were added in each well and the plate allowed to develop 30 min at 37 ºC. The absorbance was read at 562 nm.

4.2.12 NO measurement

The principle of nitrite/nitrate assay has been described in Chapter 2. The fluorometric technique, more sensitive, was chosen. Total NO was measured with a fluorometric assay kit following the manufacturer's instructions (See for details). 30 µL of each sample (culture media) or nitrate standards (diluted in BSA control solution) were transferred to a 96 well plate in duplicate, to which 50 µL of assay buffer were added. Then 10 µL of cofactors (NADPH, H^{+}) and 10 µL of nitrate reductase were added. The plate was incubated 2 h at room temperature. Then 10 µL of DAN and 20 µL of NaOH 2.8 M were added, the plate was read at λ excitation = 360 nm, and λ emission = 400 nm.

4.2.13 PGI_2 measurement

6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF1_{1\alpha}$), the stable product of PGI_2 (highly unstable) was measured by an enzyme immunoassay (EIA), or enzyme linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions. Here is the general principle of the competitive method of ELISA used here:

1- Plates are precoated with a polyclonal antibody (pAb) directed against the monoclonal Ab (mAb) added in 2

2- Free Ag (either standard or sample), labelled Ag (tracer-Ag, fixed concentration), and a mAb (fixed concentration) specific for the Ag are incubated with pAb
3- The plate is washed to remove all the compounds that did not specifically bind to the pAb.
4- The plate is revealed with a substrate for the tracer, giving a signal of either absorbance or fluorescence that is proportional to the amount of tracer-Ag, thus inversely proportional to the bound free Ag.

Briefly, 50 µL of BSA solution (diluent) and then 50 µL of sample or 6-keto $PGF1_{1\alpha}$ standard were transferred to the precoated 96 well plate. Then 50 µL of $PGF1_{1\alpha}$ tracer and 50 µL of $PGF1_{1\alpha}$ antiserum were added to each well (final volume: 200 µL), and the plate was incubated 18 h at 4 °C. It was then washed 5 times with PBS, revealed with 200 µL Ellman's reagent (2 h on an orbital shaker, room temperature) and read at 412 nm.

Standards were transferred in triplicate, while samples (2 dilutions: 1/10 and 1/100) were transferred in duplicate.

4.2.14 eNOS and COX-2 expression: Western blotting

Western blot consisted of the analysis of samples from cells treated with the 8 different FA profiles and BSA control, in absence of TNFα or insulin. For each run, protein extracts from cell culture triplicates were loaded on 3 different gels, so that the 9 profiles are visible on each membrane. The experiment was repeated on samples from 4 different sets of cell cultures for eNOS expression. Another Western blot was run on 2 sets of cultures for COX-2 expression. The general protocol is described in Appendix XXIV.

4.2.14.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins from cell lysates were separated on SDS-10% polyacrylamide gel in order to identify eNOS (~140 kDa), COX-2 (~55 kDa), as well as the house keeping gene α-tubulin (~70 kDa). SDS is a denaturant that gives a uniform negative charge to proteins which are then separated according to their molecular weight only. A 10% concentration of polyacrylamide was chosen in order to identify proteins of relatively high MW (eNOS, 140 kDa) while keeping a gel that is not too fragile.

The resolving and stacking gels were prepared according to the concentrations described in Table 4-3. After the resolving and then stacking gels were set, the comb was removed and the wells were rinsed with tank buffer. 30uL of cell lysates and 3µL 2-mercaptoethanol in 0.02% BPB were loaded in each well. Samples were run in tank buffer (Tris/glycine/SDS) at a voltage of 150 V for approximatively 1 hour and 10 min.

Table 4-3: Composition of resolving and stacking gels for SDS-PAGE

	Quantity for 3 gels	*Final concentration*
Lower gel (resolving gel)	*Total = 28.8 mL*	
4X Tris-SDS pH 8.8	7.2 mL	1X (375mM Tris0.1% SDS)
30% Acrylamide	9.6 mL	10%
Ultrapure water	12 mL	
10% APS	115 µL	0.4%
TEMED	23 µL	0.08%
Upper gel (stacking gel)	*Total = 7.92 mL*	
4X Tris-SDS pH 6.8	2 mL	1X (125mM Tris0.1% SDS)
30% Acrylamide	1.04 mL	4%
Ultrapure water	4.88 mL	
10% APS	40 µL	0.5%
TEMED	5 µL	0.06%

*APS and TEMED, initiating polymerisation, were added at the very end of the preparations. Their volumes may be adapted for a faster polymerisation.

4.2.14.2 Transfer of proteins

PVDF membranes were activated in methanol for 15s and, in order to make the membrane more hydrophilic. Membranes, gels and filters were then equilibrated in transfer buffer for a few minutes and disposed as described in Figure 4-6. Proteins were transferred for 2h at 20V for eNOS, 1h30 for COX-2.

Figure 4-6: Assembly for electrophoretic transfer of proteins for Western blot.
PVDF: Polyvinylidene fluoride

4.2.14.3 Protein detection

After transfer, gels were incubated overnight at RT in staining solution, and then rinsed 3 to 4 times in destain solution (until protein bands appear) in order to check the quality of the migration.

Membranes were incubated for 1h at RT in blocking solution, then rinsed 3 times and washed with PBS-Tween 0.1% (1x15min, 3x5min). They were then incubated with eNOS or COX-2 antibody solution overnight at 4°C, rinsed and washed as before. Next, membranes were placed in anti-rabbit or anti-mouse antibody solution for 1h at RT and then kept in PBS-Tween 0.1% for a few minutes prior to development. Fluid excess was removed and the membranes placed on plastic wrap. The enhanced chemilumescent (ECL) reagents were mixed as described by the manufacturer and applied to the membranes for 1min. Membranes were then carefully covered with the plastic wrap and visualised with the SynGene software (Chemi Sample programme, exposure time = 30min). Immediately after reading, membranes were rinsed and washed in PBS-Tween 0.1% as described earlier. They were incubated with α-tubulin antibody for 1h at RT, rinsed and washed again, and incubated for another hour at RT with anti-rat antibody solution. Membranes were then treated with ECL reagents as described above and visualised with the SynGene software (Chemi Sample programme, exposure time = 2.5 min).

4.2.1 Statistical analysis

The term FA profile is used to define the BSA, SFA, MUFA, n-6 PUFA and n-3 PUFA profiles. The term treatment characterises insulin and/or TNFα. The term FA level defines the two concentrations of FA used, i.e. 400 and 1000 µM.

For viability tests, data were analysed by one way ANOVA to compare the effect of the nine media separately (BSA control, SFA N, SFA H, MUFA N, MUFA H, *n*-6 PUFA N, *n*-6 PUFA H, *n*-3 PUFA N, *n*-3 PUFA H), without insulin or TNFα treatment.

NO and PGI_2 results were analysed by three-way ANOVA, in order to examine the effct of FA profiles, FA levels and treatment, and the interaction between factors. In this model the FA profiles were compared between them, excluding the BSA control as it was applied at only one concentration (no factor level). Subsequently, the FA level factor was excluded from the statistical model when it had no effect on NO or PGI_2 production. A two-way ANOVA was then performed (factors: FA profiles, treatment) and the effect of FA profiles could be compared to the BSA control. eNOS and COX-2 expression was analysed by two-way ANOVA, with FA levels and FA profiles as factors, excluding the BSA control from the dependent variables. When FA levels had no effect, a one-way ANOVA was performed with FA profile as a unique factor in order to compare the FA profiles with BSA control. Bonferroni's multiple comparison was used for post hoc analysis.

Normality of the residuals was checked for analysis and log transformation of the data was attempted when required. If the residuals of the log-transformed data were still not normally distributed for the one-way ANOVA analysis, non-parametrical analysis was performed (Kruskal-Wallis). Non parametric tests were not required to substitute two- and three-way ANOVA. Results are shown as means (SEM). All statistical analysis was carried out with SPSS for Windows (version 17.0, SPSS, Chicago, IL).

4.3 Results

As described in 4.2.4, cell culture work was divided in 4 steps:

1- Preparation of the FA profiles

2- Testing the media to apply to the cells (viability and endotoxin tests)

3- TNFα and insulin levels determination

4- Assessment of the effect of FA profiles on EF

4.3.1 FA composition of FA profiles

The total FA concentration measured by GC analysis for each of the 8 profiles showed good compliance with the expected total FA levels (400 and 1000 μM), varying from 96.2 to 98.2% of the expected total concentrations (results not shown). The composition of each profile was also shown to conform to the expected percentages, as shown in Table 4-4. The percentages measured in all profiles represented 98.8 % +/- 9.3% (SD) of the expected values. n-3 PUFA levels in the SFA, MUFA and n-6 PUFA profiles fell below the detection limit of the GC for the 'normal' FA profiles (400 μM) but concentrations measured in the 'high' FA profiles (1000 μM) were consistent with expected 2%.

Table 4-4: FA percentages obtained in the high SFA-, high MUFA-, high n-6 PUFA- and high n-3 PUFA-profiles, measured by gas chromatography

FA profiles		Molar percentages of FA measured, *expected*			
		Total SFA	Total MUFA	Total n-6 PUFA	Total n-3 PUFA
SFA	FA "High"	52.8	32.4	12.7	2.1
	FA "Normal"	57.5	31.2	11.3	n.d.
	Expected	*55.0*	*30.0*	*13.0*	*2.0*
MUFA	FA "High"	38.9	48.5	10.8	1.8
	FA "Normal"	43.0	47.2	9.8	n.d.
	Expected	*35.0*	*50.0*	*13.0*	*2.0*
n-6 PUFA	FA " High "	34.1	36.7	27.1	2.0
	FA "Normal"	38.6	35.1	25.7	n.d.
	Expected	*35.0*	*35.0*	*28.0*	*2.0*
n-3 PUFA	FA " High "	34.1	36.7	12.6	16.7
	FA "Normal"	36.0	36.3	11.4	16.3
	Expected	*35.0*	*35.0*	*13.0*	*17.0*

FA, fatty acids; SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; n.d., not detected.

4.3.2 Viability and endotoxin tests

4.3.2.1 Endotoxin Assay

- *Qualitative measurements*

All the FA-BSA mixtures before complexation (BSA:FA 2.4 mM : 6 mM) showed a positive result to the endotoxin test, showing an endotoxin concentration > 0.125 EU/mL (Table 4-5).

Table 4-5: Results of the endotoxin test on FA-BSA mixtures

Standard [ETX] EU/mL	0.5	0.25	0.125	0.063	0.031	0.016
Results	+	+	+	-	-	-

FA-BSA mixtures	PA	SA	OA	LA	AA	EPA	DHA
Results	+	+	+	+	+	+	+

ETX: endotoxin, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

As the effects of endotoxin on endothelial function were showed with high concentration of endotoxin (from 10 ng/mL, i.e. from 100 EU/mL), a semi-quantitative measurement was performed, using 10 dilutions of BSA solution (control), to see if the real content of endotoxin was in the range of concentration that can affect endothelial function *in vitro*.

- *Semi-quantitative measurement (BSA control, several dilutions)*

The second test showed that the BSA control solution used in the culture (~40 mg/mL) solution contained endotoxin at a level comprised between 2.5 and 50 EU/mL, (Cf. Table 7, see below for details of calculation) equivalent to 0.25 to 5 ng/mL.

Table 4-6: Results of the endotoxin (ETX) test on 10 dilutions of BSA

Standard [ETX] EU/mL	0.5	0.25	0.125	0.063	0.031	0.016
result	+	-	-	-	-	-

Dilution of BSA	1	10	100	1000	2000	4000	8000	16000	32000
result	+	+	-	-	-	-	-	-	-

Details for calculation:

- The ETX standards showed a positive result at 0.5 EU/mL and a negative result at 0.25 EU/mL. Thus the detection limit of the test was 0.5 EU/mL, i.e. 0.05 ng/mL.

- The samples showed a postive results at dilution 1/10: $[ETX]_{1/10} > 0.25$ EU/mL, i.e. $[ETX]_1 > 2.5$ EU/mL in the BSA control. No gel was observed for the dilution 1/100: $[ETX]_{1/100} < 0.5$ EU/mL, i.e. $[ETX]_1 < 50$ EU/mL.

4.3.2.2 MTT test

As FA and BSA were complexed with a same molar ratio in both high and normal FA profiles, they had different BSA levels and it was initially intended to use two BSA controls, characteristic of the two FA levels. The viability of the cells was not affected by the different media tested ($P = 0.682$, one way ANOVA) (Figure 4-7).

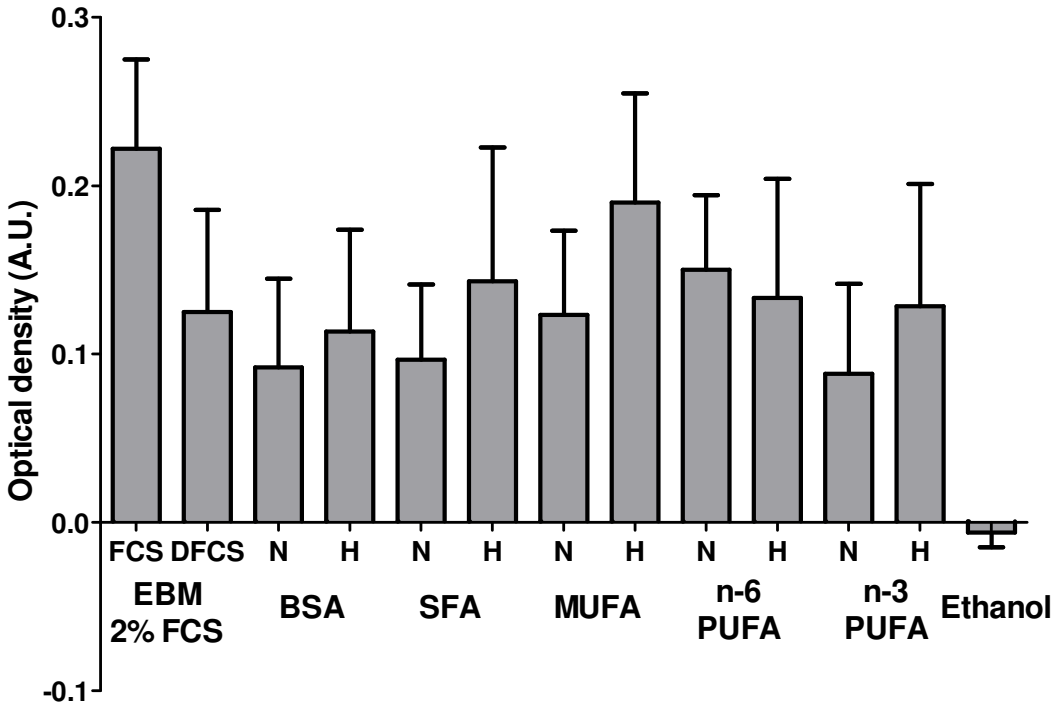

Figure 4-7: Cytotoxicity of the FA profiles in HDMEC

HDMEC were incubated with 24 h with either Endothelial basal medium (EBM) – 2% fetal calf serum (FCS, resting medium), EBM 2% delipidised FCS (DFCS), BSA only, or fatty acid (FA) – BSA profiles of 4 different compositions: enriched in saturated FA (SFA), in monounsaturated FA (MUFA), in n-6 polyunsaturated FA (PUFA) n-3 PUFA. These profiles were applied at either 400 (Normal, N) or 1000 µM FA (High, H). Two levels of BSA were used: 16.62 +/- 0.6 (N) and 41.55 +/- 1.5 (H) mg/mL. The experiment was repeated 6 times in triplicate.

In order to rule out the effect of BSA levels may on endothelial function, BSA was added to all the low FA profiles to reach the same amount of BSA as the high FA profile. Thus, the average of BSA that was used as a unique control in all the next experiments was the average of BSA contained in each profile, i.e. 41.55 mg/mL, which is in the range of physiological serum albumin concentration in human (30-50mg/mL). This allowed the results to be expressed in percentage relative to the unique BSA control.

4.3.2.3 LDH assay

The LDH assay was performed twice in four replicates on FA profiles containing similar levels of BSA (from 39.8 to 42.9 mg/mL). The average of BSA

levels of these profiles was used as a unique control (41.55 mg/mL), which is in the range of physiological serum albumin concentration in human (30-50mg/mL). This control was used for all the next experiments and allowed the expression of the data relative to the BSA control. There was no significant difference in toxicity between the 9 media tested (P = 0.926, Kruskal Wallis). However LDH activity appeared slightly higher in the high FA media, corresponding to a slightly higher amount of apoptotic and/or necrotic HDMEC (Figure 4-8). Because cell growth may slightly vary between wells, NO and PGI_2 measurements were readjusted to the number of cells by expressing results as per µg protein.

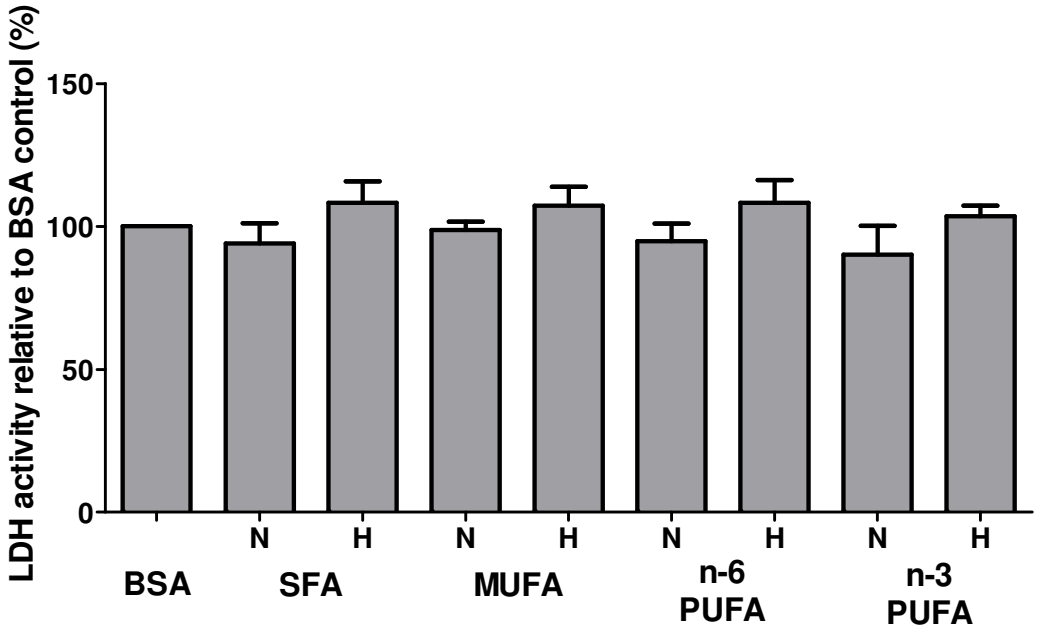

Figure 4-8: Assessment of FA profiles cytotoxicity on HDMEC by LDH assay
HDMEC were incubated with 24 h with either BSA only, or fatty acid (FA) – BSA profiles of 4 different compositions: enriched in saturated FA (SFA), in monounsaturated FA (MUFA), in n-6 polyunsaturated FA (PUFA) n-3 PUFA. These profile were applied at either 400 or 1000 µM FA, corresponding to normal (N) or high (H) levels, respectively. The average level of BSA was 41.55 mg/mL +/- 1.5. The experiment was done twice in 2 replicates.

4.3.3 Determination of TNF and insulin levels to be used

NO concentrations were either very low or not enough to be detected, making the optimisation of insulin and TNF α impossible. Despite having no cytotoxic effect on HDMEC, the absence of lipid in the FFA-free control (BSA only) profiles (using delipidised FCS and delipidised BSA) may have compromised the stability and function of the cells, and possibly affected NO production.

The final volume of medium was reduced to 300 µL per well in order to increase the NO concentration and make it detectable by the fluorometric assay. TNFα (210-TA-010, R&D Systems) and insulin (4502-01, Millipore) concentrations were chosen according to the manufacturer's instructions.

4.3.4 Effects of FA profiles, insulin and TNFα on EF

4.3.4.1 NO production

- *Three-way ANOVA (BSA control excluded)*

NO concentrations were generally very low and showed high standard deviations between triplicate wells and repeat experiments (n = 5). There was no interaction between treatment, FA profile and/or FA levels. There was no main effect of treatment (P = 0.595) or FA level (P = 0.209) but a borderline significant effect of FA profile (P = 0.045, three-way ANOVA, factors = FA profiles, FA levels, insulin/TNFα treatment) due to NO levels ~two fold higher upon SFA profile compared to n-3 PUFA profile (P = 0.032, Bonferroni's multiple comparison).

- *Two-way ANOVA (BSA control included)*

Because FA level had no effect on NO production, a two-way ANOVA was performed with FA profile and treatment as factors. There was no interaction between FA profile and treatment and there was no effect of treatment. There was a borderline significant main effect of profile (P = 0.049) and NO was significantly higher with SFA than n-3 PUFA (P = 0.042, Bonferroni's multiple comparison), differences between all the other profiles, including compared to the BSA control (no FA) did not reach significance (two-way ANOVA, factors = FA profiles, insulin/TNFα treatment) (Figure 4-9).

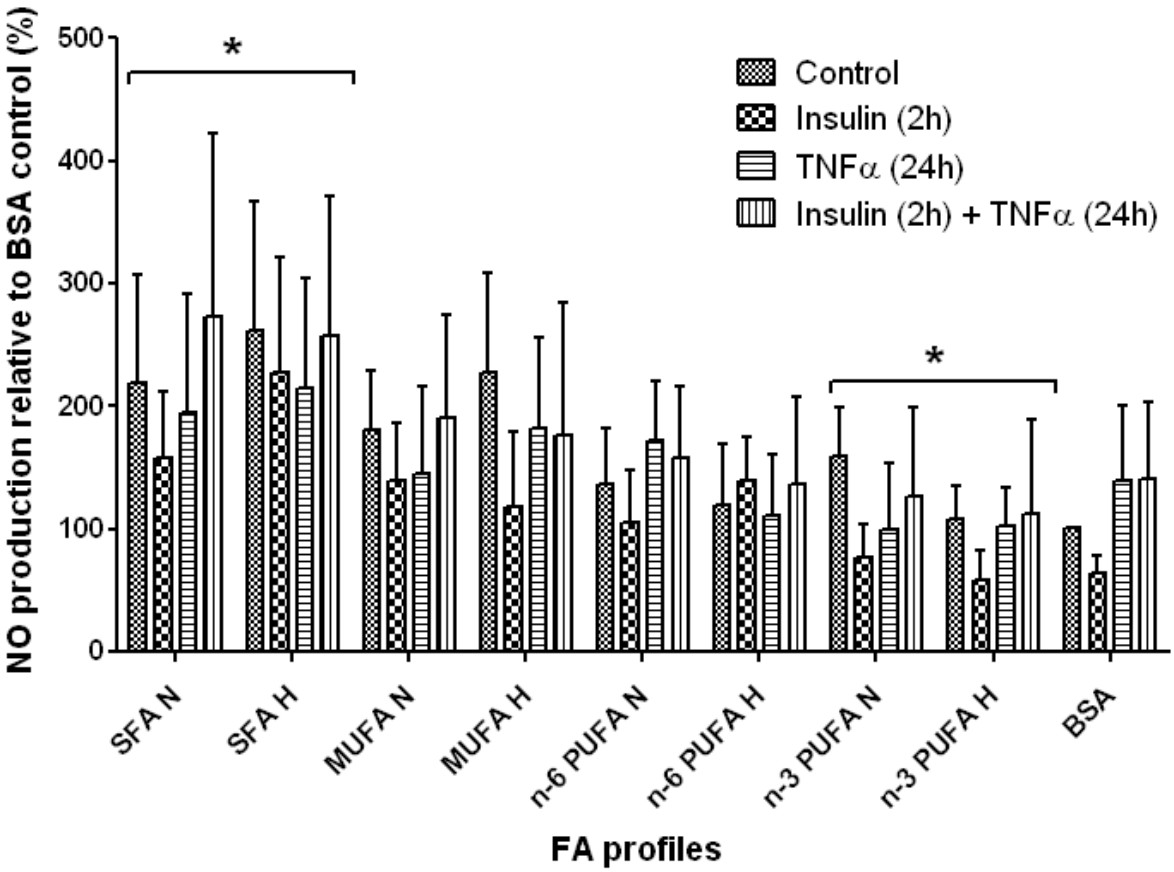

Figure 4-9: Effect of different FA profiles at high (H, 1000 µM) and normal (N, 400 µM) levels upon insulin (2h) and/or TNFα (24h) treatment on NO production in HDMEC

Means are expressed as percentage of NO production adjusted for protein amount relative to BSA control (no FA, no insulin or TNFα). Error bars are SEM. HDMEC were incubated 24h in the presence of each FA profile (BSA-EBM-delipidised FCS 2%), in the presence or absence of TNFα 10 ng/ml for 24h and/or insulin 10 µg/mL for the last 2h.

* $P = 0.042$ between SFA and n-3 PUFA profile (two-way ANOVA; factors = FA profiles, treatment).

4.3.4.2 PGI_2 production

- *Three-way ANOVA (BSA control excluded)*

There was no main effect of FA level ($P = 0.242$) but a significant effect of FA profile ($P < 0.001$) and treatment ($P < 0.001$). TNFα significantly increased 6-keto $PGF_{1\alpha}$ in all FA profiles regardless of the level applied ($P < 0.01$), while insulin had no effect. 6-keto $PGF_{1\alpha}$ concentrations were increased by n-6 PUFA compared to SFA ($P = 0.001$), MUFA ($P = 0.011$) and n-3 PUFA ($P < 0.001$) while they did not differ between MUFA and SFA profiles. n-3 PUFA showed lower levels of 6-keto $PGF_{1\alpha}$ compared to the three other FA profiles ($P < 0.001$). There was no interaction between factors (three-way ANOVA, factors = FA profiles, FA levels and insulin/TNFα treatment).

- *Two-way ANOVA (BSA control included)*

Because FA levels had no effect on 6-keto $PGF_{1\alpha}$ concentrations, it was removed from the statistical model. As for the three-way ANOVA, the two way ANOVA analysis showed no interaction between factors and a significant effect of treatment ($P < 0.001$) and FA profile ($P < 0.001$). The differences between treatments and between the FA profiles were the same as those observed with the three-way ANOVA model, with similar significance: PGF1α levels were significantly increased upon TNFα treatment ($P = 0.002$) but remained unchanged by insulin. 6-keto PGF1α levels were higher with n-6 PUFA compared to SFA ($P = 0.001$), MUFA ($P = 0.013$) and n-3 PUFA ($P < 0.001$) and did not differ between SFA and MUFA. Compared to BSA control (no FA), 6-keto $PGF_{1\alpha}$ were higher with SFA ($P = 0.003$), MUFA ($P < 0.001$) and n-6 ($P < 0.001$) but unchanged by the n-3 PUFA profile (two-way ANOVA, factors = FA profiles and insulin/TNFα treatment) (Figure 4-10).

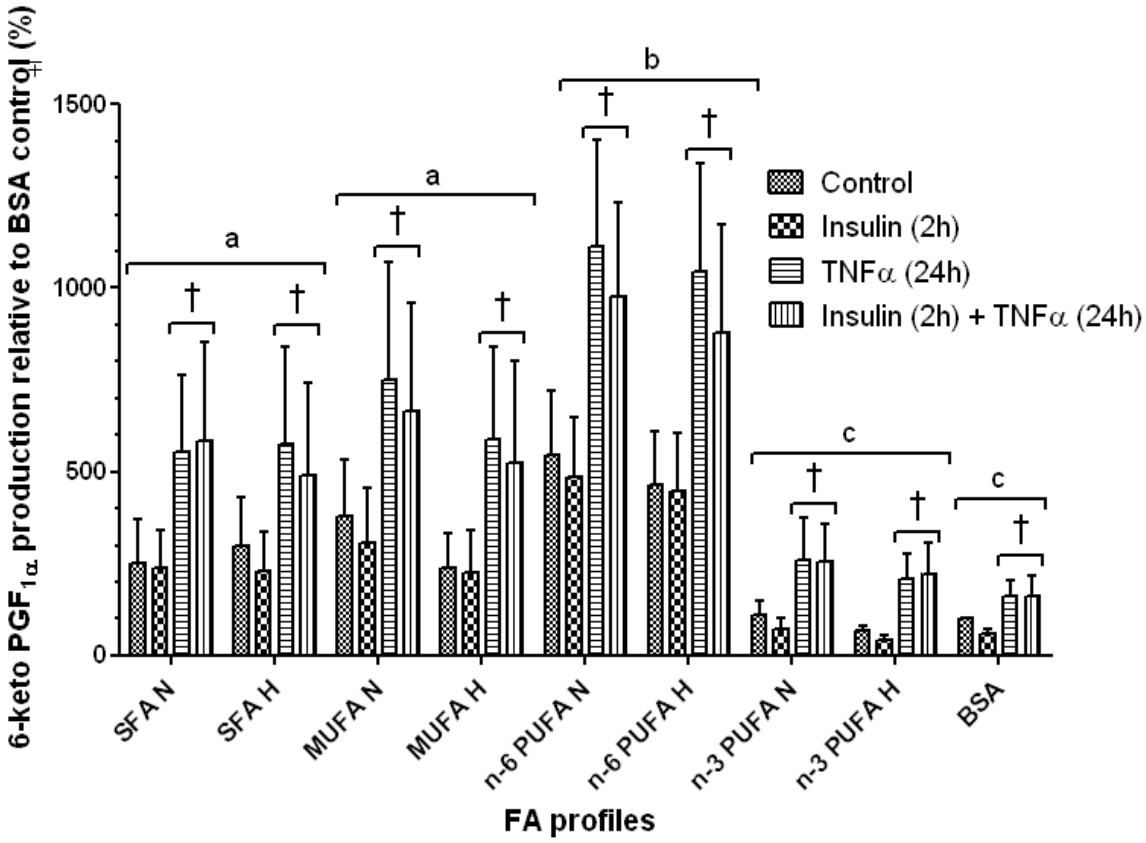

Figure 4-10: Effect of different FA profiles at high (H, 1000 µM) and normal (N, 400 µM) levels upon insulin (2h) and/or TNFα (24h) treatment on PGI_2 production in HDMEC

Means are expressed as percentage of 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) production adjusted for protein amount relative to BSA control (no FA, no insulin or TNFα). Error bars are SEM. The experiment was repeated 5 times. HDMEC were incubated 24h in the presence of each FA profile (BSA-EBM-delipidised FCS 2%), in the presence or absence of TNFα 10 ng/ml for 24h and/or insulin 10 µg/mL for the last 2h.

† TNFα increased overall $PGF_{1\alpha}$ levels compared to control ($P = 0.002$) while insulin had no significant effect. There was no treatment*profile interaction. Different letters show significantly different $PGF_{1\alpha}$ production upon the different FA profiles ($P < 0.02$, two-way ANOVA, factors = FA profiles and insulin/TNFα treatment).

4.3.4.3 eNOS expression

As for NO concentrations, eNOS was generally very low and showed a high standard deviation between triplicate wells and repeat experiments (n = 4) (see below). There was no effect of FA profiles or FA levels on eNOS expression, and no interaction between factors (two-way ANOVA, factor = FA profile, FA level). When the factor level was excluded from the model, eNOS expression did not vary between the different profiles including the FA free BSA control (One-way ANOVA, factor = FA profile).

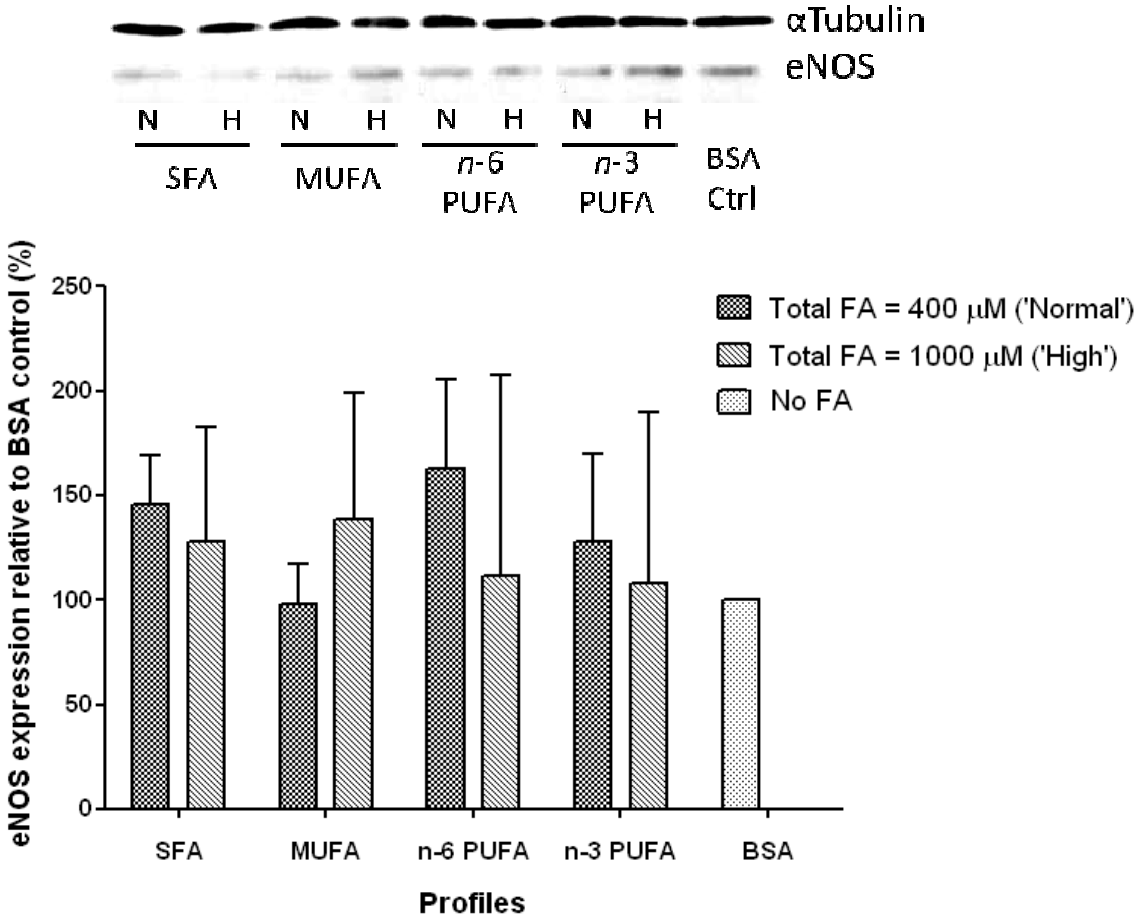

Figure 4-11: Effect of different FA profiles at normal (N, 400µM) and high (H, 1000µM) level on eNOS expression in HDMEC, in absence of treatment (insulin and/or TNFα)

Top: Sample of Western Blot analysis of eNOS and housekeeping gene (α-tubulin) expression

Bottom: Means are expressed as percentage of eNOS expression adjusted for α-tubulin expression, relative to BSA control (no FA, no insulin or TNFα). Error bars are SEM. The Western Blot was run from 4 sets of experiments in triplicate (n = 12). HDMEC were incubated 24h in the presence of each FA profile (BSA-EBM-delipidised FCS 2%), at 400µM and 1000µM concentration, in the absence of TNFα and/or insulin.

4.3.4.4 COX-2 expression

- *Two-way ANOVA analysis (BSA control excluded)*

When BSA control was excluded from the model, there was no effect of FA profile or level on COX-2 expression, and no interaction between factors (Two-way ANOVA; factors = FA profiles, FA levels).

- *One-way ANOVA (BSA control included)*

In order to compare the FA profiles with BSA control, the factor 'FA level' was excluded from the statistical model. All FA profiles reduced COX-2 expression compared to BSA control (no FA) ($P < 0.001$) (One-way ANOVA, factor = FA profiles, Bonferroni's multiple comparison) (Figure 4-12).

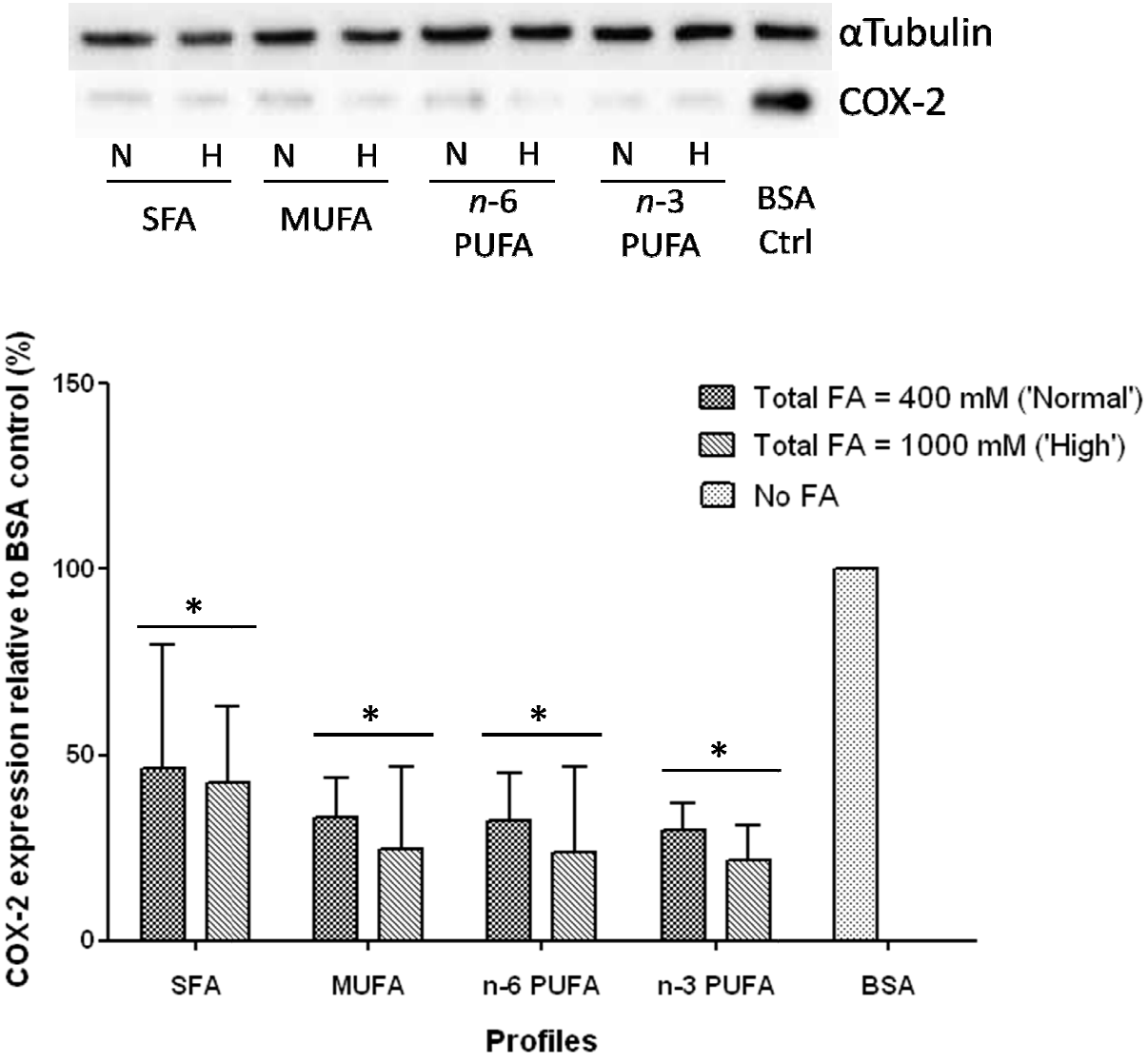

Figure 4-12: Effect of different FA profiles at normal (400µM) and high (1000µM) level on COX-2 expression in HDMEC, in absence of treatment (insulin and/or TNFα).

Top: Sample of Western Blot analysis of COX-2 and housekeeping gene (α-tubulin) expression

Bottom: Means are expressed as percentage of COX-2 expression adjusted for α-tubulin expression, relative to BSA control (no FA, no insulin or TNFα). Error bars are SEM. The Western Blot was run from 2 sets of experiments in triplicate (n = 6). HDMEC were incubated 24h in the presence of each FA profile (BSA-EBM-delipidised FCS 2%), normal (400µM) and high (1000µM) concentration, in the absence of TNFα and/or insulin.

* $P < 0.001$ compared to BSA control (One-way ANOVA; factor = FA profiles)

4.4 Discussion

The primary hypothesis for this endothelial cell culture study was that NO production would be inhibited by a fatty acid profile enriched in SFA and increased by profiles enriched in *n*-3 PUFA. NO production was low in all profiles, yet slightly increased with SFA compared to *n*-3 PUFA, while it remained unchanged by insulin and TNFα, eNOS expression remained low and unchanged in all profiles. It was also hypothesised that PGI_2 would be increased upon treatment with an *n*-6 PUFA-enriched fatty acid profile, and decreased by an *n*-3 PUFA rich profile. This was proven, while COX-2 expression remained unchanged in all FA profiles and lower than with BSA control. It was also expected that insulin-induced NO production would be inhibited by the high-SFA profile and stimulated by the *n*-3 PUFA profile, and that an inflammatory stress-induced reduction in NO was expected to be ameliorated by *n*-3 PUFA profiles. This was not observed since insulin and TNF-α did not affect NO production. Furthermore, the level of total FA used, either 400 or 1000 μM, as observed in healthy and insulin resistant state, respectively, did not affect NO and PGI_2 production or eNOS and COX-2 expression.

The BSA-FA complexation steps, as well as the freezing did not affect the FA concentrations in the different FA profiles tested, which correspond to the levels and percentages we expected, and all fall within the physiological range. SFA and MUFA reached ~50-55% in the SFA and MUFA profiles, respectively. Total PUFA constituted 15% of the total FA in the SFA and MUFA, while it reached ~30% in the high PUFA profiles. The n-6:n-3 ratio was ~5:1 in the SFA and MUFA profiles while it reached 14:1 in the n-6 PUFA profile, corresponding to the high n-6 PUFA intake observed in the western populations. These proportions enclose the physiological ranges previously observed in NEFA for each category (Conquer, et al., 2002, Conquer, et al., 1999, Conquer and Holub, 1998, Frape, et al., 2000, Newens, et al., 2011, Rhee, et al., 2008). In the n-3 profiles, the n-6:n-3 ratio was ~1:1.3, as it was estimated that n-6 and n-3 PUFA were equivalent in the human diet and thus in circulating FA before the industrial and agricultural revolution (Simopoulos, 1999).

4.4.1 Nitric oxide and eNOS expression

NO concentrations and eNOS expression were generally very low and showed high standard deviations between triplicate wells and repeat experiments, suggesting that HDMEC have a very low basal expression, and that the NO assay was not sensitive enough to detect changes in NO production in this cell type. There are important phenotypic differences between endothelial cells from large vessels and the microvascular EC isolated from different tissues and organs (Kumar, et al., 1987), which may explain the low levels observed in our study. In contrast with most macrovascular EC, cardiac microvascular EC from rat were reported to have no basal expression of eNOS, while being able to express substantial amount of iNOS upon inflammatory stimulation (Balligand, et al., 1995). Ando *et al.* later reported lower eNOS expression and NO production in porcine coronary microvascular EC compared to aortic EC (Ando, et al., 1999). Nonetheless, previous findings suggest that HDMEC express eNOS and produce NO in basal conditions . Although NO or eNOS expression has not been measured directly in HDMEC, previous work has reported basal uptake of L-Arginine (eNOS substrate) as well as eNOS activity (conversion of arginine to citrulline) (Xiao, et al., 2001). The activity of eNOS is subject to complex regulations, involving phosphorylation on specific residues, binding to the calcium-calmodulin complex, the presence of cofactors of oxidoreduction and co- and post translational modifications (Sessa, 2004, Zhang, et al., 2011). The latter involve acylation – especially myristoylation and palmitoylation – that are essential for targeting eNOS to caveolae, a necessary step for eNOS activation and optimal NO release (Liu, et al., 1996, Shaul, et al., 1996). The caveolae is a particular type of lipid raft whose composition may greatly influence eNOS activity (Chikani, et al., 2004). It is likely that the lack of lipids in the FA-free BSA control compromised this step in the activation of eNOS, making the set up of TNFα and insulin concentrations impossible.

4.4.1.1 Basal NO production and eNOS expression with different FA profiles

Surprisingly, only the addition of SFA profiles appeared to slightly – yet significantly – increase NO production, compared to *n*-3 PUFA, in which NO production remained similar to the FA-free BSA control. As mentioned earlier, palmitoylation of eNOS is crucial for targeting eNOS to caveolae and its activation (García-Cardeña, et al., 1996, Goligorsky, et al., 2002). Therefore, the enrichment of the SFA profiles in palmitic acid (37%) compared to the other profiles (23%) may have

stimulated the palmitoylation of eNOS and explain the increase of NO production observed upon SFA profile, while eNOS remained unchanged. Consistent with our findings, previous research has reported stimulation of eNOS activity by palmitic acid complexed to serum albumin, and to a greater extent myristic acid (C14:0, absent of the FA profiles used in our study), compared to other individual FA including stearic, oleic and linoleic acid, in human microvascular EC (Zhu and Smart, 2005), as well as EA.hy926 (macrovascular EC, immortalised) (Couloubaly, et al., 2007). This effect was shown to be mediated through the AMP kinase pathway, rather than the PI3K pathway or intra-cellular calcium increase (Zhu and Smart, 2005).

The reason why *n*-3 PUFA failed to improve basal NO production, which remained significantly lower than with SFA, remains uncertain and does not prove our hypothesis. While *n*-3 PUFA, including EPA and DHA have been widely shown to stimulate NO-dependent vasorelaxation *in vivo* and *ex vivo* (Engler and Engler, 2000, Lawson, et al., 1991, Omura, et al., 2001), their effect on eNOS and NO production in EC *in vitro* remains controversial. Consistent with our findings, neither EPA nor DHA affected basal eNOS activity in EA.hy926 cells (Tardivel, et al., 2009), despite an increase in basal phosphorylation of eNOS (Gousset-Dupont, et al., 2007). In contrast, several studies have shown that either EPA or DHA stimulated basal NO production and eNOS activity in various other cell types, including HUVEC (Chisaki, et al., 2003, Li, et al., 2007, Li, et al., 2007, Okuda, et al., 1997, Omura, et al., 2001), as well as human coronary artery EC (Stebbins, et al., 2008) and retinal microvascular EC (Matesanz, et al., 2010). This beneficial effect was attributed to the enrichment of *n*-3 PUFA in caveolae, which coincided with the displacement of Cav-1 from the lipid raft and the translocation of eNOS to the cytosol (Li, et al., 2007, Li, et al., 2007, Matesanz, et al., 2010). It should be noted that this was shown for incubation times of 24-48h, which is sufficient for EPA or DHA to be incorporated into the membrane and reach a constant equilibrium (Gousset-Dupont, et al., 2007). When applied for a shorter period (10-60 min), LC n-3 PUFA may exert their effect through different mechanisms, such as the stimulation of calcium inflow through the cell membrane (Chisaki, et al., 2003, Okuda, et al., 1997). The mechanisms may also vary depending on the dose and cell type used, which may contribute to the discrepancies observed and explain the lack of effect of n-3 PUFA in our experiments as well as others (Gousset-Dupont, et al., 2007, Tardivel, et al., 2009). Importantly, the studies mentioned above have investigated the effect of FA individually, rather than as a combined mixture as observed in circulating

NEFA *in vivo*, and sometimes refer to a control containing a different FA, such as arachidonic or palmitic acid (Matesanz, et al., 2010), which could interfere with eNOS activity and trafficking, and thus be questioned as a control.

Notably, saturated FA, especially palmitic acid, were also reported to stimulate the inflammatory transcription factor the nuclear factor (NF-) κB in several types of human EC, in contrast to unsaturated fats such al oleic, linoleic or palmitoleic acid (Cacicedo, et al., 2004, Staiger, et al., 2006). NF-κB is involved in the activation of iNOS (Forstermann and Kleinert, 1995) and may have contributed to the increase in NO production observed upon SFA profiles. In contrast *n*-3 PUFA, especially DHA, have been widely shown to inhibit inflammatory processes (De Caterina, et al., 1994), and suppress NF-κB nuclear translocation and upstream I-κBα phosphorylation and degradation in human EC (Chen, et al., 2005, Wang, et al., 2011). Thus the pro- and anti-inflammatory actions of SFA and of *n*-3 PUFA, respectively, may be responsible for opposite effects on iNOS activity, which would explain the significant difference in NO production upon these two profiles, while eNOS remained unchanged.

4.4.1.2 NO production and eNOS expression following insulin and TNFα treatment

Both macro- and microvascular endothelial cells express insulin receptors (Jialal, et al., 1985), and the binding of insulin activates the PI3K pathway, which mediates the activation of eNOS as well as an anti-apoptotic effect (Hermann, et al., 2000, Kuboki, et al., 2000). Surprisingly, insulin failed to increase NO production in HDMEC, even in the presence of the various FA profiles. This contrasts with previous research in HDMEC where insulin was able to increase tyrosine phosphorylation of the insulin receptor and its substrate IRS-1, leading to the activation of protein kinase B, proteins involved in the activation of eNOS through the PI3K pathway (Gogg, et al., 2009). However this study showed a low expression of insulin receptors and previous work has shown that HDMEC were resistant to insulin at the receptor level (Johansson, et al., 2008), which may explain the lack of insulin on NO production in our study. Pro-inflammatory cytokines, including TNFα, have been widely reported to down regulate basal and insulin mediated eNOS expression and activity in EC (Kim, et al., 2001, Lai, et al., 2003, Zhang, et al., 1997), including human microvascular EC (Li, et al., 2007, Neumann, et al., 2004). This effect was not observed in our study, which is likely to be due to the low levels of NO observed in basal conditions and upon insulin treatment.

Interestingly, saturated fats, and particularly palmitic acid have been shown to impair insulin-induced eNOS activation in human and bovine aortic EC (Kim, et al., 2005, Wang, et al., 2006), as well as human microvascular EC (Kim, et al., 2007). In contrast *n*-3 PUFA have been reported to modulate eNOS activity induced by vasoactive coumpounds such as insulin (Madonna, et al., 2011) or histamine (Gousset-Dupont, et al., 2007, Tardivel, et al., 2009), and lysophosphatidylcholine (Tardivel, et al., 2009). Because HDMEC did not respond to insulin or TNFα, the effect of SFA and n-3 PUFA profiles on insulin-induced eNOS activation and NO production or inflammatory processes could not be observed.

4.4.2 PGI2 and COX-2 expression

4.4.2.1 PGI_2 and FA profiles

The increased PGI_2 upon *n*-6 PUFA compared to the other profiles is likely to be due to the higher arachidonic acid (AA) levels in this profile. After its release from TAG, DAG or PL through phospholipase, AA is incorporated into the EC, where it is converted into PGI_2 through the combined actions of PGH_2 synthase (cyclooxygenase 1 or 2) and prostacyclin synthase (PGIS, cytochrome P450 isomerase) (Gryglewski, 2008). As a substrate for PGI_2 production, AA dose dependently stimulates the formation of PGI_2 by COX-2 in various cell types including HUVEC, as well as aortic macrovascular and microvascular EC (Egan and FitzGerald, 2006, Goldsmith, et al., 1981, Johnson, 1980, Spector, et al., 1983). It should be noted that linoleic acid (LA) was present in higher proportions than AA in the n-6 PUFA profile and it could be hypothesized that conversion from LA to AA contributed to the increase in PGI_2 production (Salem, et al., 1999). However this process appears limited in EC (Grammatikos, et al., 1994, Kaduce, et al., 1982) and LA has actually been reported to decrease the production of PGI_2, *in vivo* and *in vitro* (Batres-Cerezo, et al., 1991, Brox and Nordoy, 1983, Umeda, et al., 1990). Therefore, the increase of AA (3%), as well as the AA/LA ratio (~1:8) in the n-6 PUFA profile compared the other profiles (AA = 1% , AA/LA ~ 1:12), rather than an increase in total *n*-6 PUFA *per se*, is therefore more likely to explain the PGI_2 increase observed. Surprisingly, COX-2 levels did not follow PGI_2 production but they remained low compared to BSA control and and there were no significant differences between FA profiles. COX-2 expression may have been upregulated by AA at the beginning of the incubation, leading to the accumulation of 6-keto $PGF1_{1\alpha}$, stable metabolite of PGI_2, and returned to a lower basal level by the end of the 24h incubation. The higher level of COX-2 expression following the BSA control

treatment is probably due to the delipidated environment which may have stressed the cells.

n-3 PUFA profiles reduced PGI_2 production compared to SFA and MUFA profiles, despite containing the same proportions of AA (1%). This suggests that while SFA and MUFA profiles stimulated PGI_2 production, possibly due to their AA content, *n*-3 PUFA were able to compete with AA and nullify its stimulating effect on PGI_2 production. Consistent with this, previous research has shown that both EPA and DHA – individually - decreased PGI_2 production in HUVEC (Brox and Nordoy, 1983, Oudot, et al., 1998) as well as bovine aortic EC (Benistant, et al., 1993). The mechanisms could involve the reduction of the substrate AA in cell membrane, concurrent with the increase of EPA content (Spector, et al., 1983), as well as a direct inhibition of COX-2 expression and/or binding to AA. EPA is capable of counteracting AA for its oxygenation into its derivative bioactive products, especially PGI_3, which is concurrent with the decrease in PGI_2 (Lagarde, et al., 1993, Morita, et al., 1983). Furthermore the anti-inflammatory role of EPA and DHA *in vitro* has been well established in various types of EC (De Caterina and Massaro, 2005), including human microvascular cells (Ait-Said, et al., 2003, Chen, et al., 2005, Ibrahim, et al., 2011). Both *n*-3 PUFA were reported to down regulate COX-2 expression through the suppression of NF-kappa B in HUVEC (Lee, et al., 2009).

4.5 Conclusion, limitations and future work

The strength of our study is that we used physiological FA profiles to simulate NEFA composition in different dietary conditions. Previous studies used single FA which is not representative of a physiological FA milieu in serum and may actually influence cellular responses. In addition, the microvascular model used was directly relevant to endothelial dysfunction and the associated microvascular dysfunction observed in insulin resistant states. One major limitation of our study is that HDMEC did not respond to insulin, thus the effect of NEFA on insulin-induced eNOS could not be explored. Unfortunately, the set up of TNF α and insulin concentrations was not possible, and the two treatments were applied at supra physiological levels. The FA-free BSA medium used may be questioned as a control, and n-6 PUFA profiles could be considered as an alternative control profile, in comparison with the other profiles mimicking high oily fish diet, Western diet and Mediterranean diet. In contrast, most

studies use another FA as a control, which may affect EF in vitro. We could envisage shorter periods of treatment in order to see if COX-2 and/or eNOS expression, as well as eNOS phosphorylation, have been stimulated earlier in the incubation.

To conclude, we have shown that a SFA-rich FA profile, representing average plasma NEFA compositions expected in populations consuming a "Western" style dietary patterns, slightly increased nitric oxide production compared to n-3 PUFA. Furthermore, an EPA and DHA-rich fatty acid profile, representing average NEFA compositions expected in populations consuming very high amounts of marine PUFA, such as the Inuit and other Greenland/Alaskan populations, inhibited PGI_2 production compared to the other 3 fatty acid profiles. At 24 h, eNOS and COX expression were not different between fatty acid profiles, but it is unknown whether changes might have been observed at an earlier time point, such as 4 h. The difficulties in trying to replicate physiological conditions are highlighted by this experiment. However, the results do suggest that variations in circulating NEFA profiles may directly impact on the release of vasodilators by the microvascular endothelial cells, and that they are more likely to reflect events occurring in the endothelium *in vivo* than comparable studies that have used single fatty acids. The total concentration of NEFA does not seem to impact on these observations, contrasting with previous findings suggesting that elevated NEFA inhibit eNOS activity in EC *in vitro* (Kim, et al., 2005, Wang, et al., 2006). Future studies in this area should try to replicate the findings reported here in different types of endothelial cells (aortic, for example), with an emphasis on shorter term changes over 4-8 h, and a more detailed investigation into eNOS phosphorylation status.

Chapter 5 Final discussion and conclusions

This thesis set out to test the hypothesis that dietary FA composition, influencing circulating FA pattern, affect endothelial and microvascular function in humans. On the one hand we compared the effects of two individual FA from fish oils, EPA and DHA, on novel markers of endothelial and microvascular function in humans, and related the outcomes measured to erythrocyte and NEFA composition. On the other hand, we compared the effect of four FA profiles (rich in either SFA, MUFA, *n*-6 PUFA, or *n*-3 PUFA), representative of average NEFA profiles observed in populations characterised by four different dietary patterns, on EF in HDMEC *in vitro*. We found that neither EPA nor DHA, supplied for 6 weeks at ~3 g/d, affected EPC or capillary density in the finger in healthy young males, but capillary density was directly correlated to DHA levels in erythrocytes at baseline. In HDMEC, FA profiles differentially affected the production of the two main vasodilators, namely nitric oxide (NO) and prostacyclin (PGI_2). NO production was low in all profiles but slightly increased by SFA compared to *n*-3 PUFA; PGI_2 was increased upon SFA and MUFA, and to a greater extent by *n*-6 PUFA compared to *n*-3 PUFA profile.

The novelty of our cell culture work is that we used combinations of FA representing NEFA compositions following four different dietary patterns, at physiological concentrations. In contrast, previous *in vitro* research has focused on investigating the individual effect of FA, sometimes at supra-physiological concentrations. While this approach gives great insight regarding the mechanisms involved in the regulation of EF, the extent to which this fits in the broader physiological perspective is questionable. For example, eNOS, a key enzyme involved in EF, requires targeting to a specific lipid raft in the membrane (caveolae) and subsequent dislocation to the cytosol, in which saturated fats (palmitic, myristic) as well as *n*-3 PUFA play an important role (Goligorsky, et al., 2002, Li, et al., 2007, Li, et al., 2007). Our study thus aimed at optimising the cell culture medium and see whether a mixture of FA that are found in circulating blood, formulated into 4 profiles corresponding to specific dietary patterns, could be defined in terms of their effects on production of endothelial NO and PGI_2. We showed that physiological FA profiles differently affected PGI2 production with the following order: *n*-6 PUFA > SFA/MUFA > *n*-3 PUFA. The higher content of arachidonic acid (AA) in *n*-6 PUFA profile, the anti-

inflammatory role of *n*-3 PUFA (Lee, et al., 2009), as well as their competition with AA for COX-2 (Lagarde, et al., 1993, Morita, et al., 1983, Spector, et al., 1983), are likely to explain this effect. Surprisingly, SFA profiles, characteristic of the western diet, had the most beneficial effect on NO production, which could be due to a favourable effect on eNOS or iNOS activity, through palmitoylation (Goligorsky, et al., 2002) or a pro-inflammatory action (Collins et al., 1990, MacMahon et al., 1990), respectively.

However caution should be taken when interpreting our results as NO was relatively low in all profiles, which suggests that physiological FA compositions are not ideal for EF *in vitro*, despite being apparently harmless to the cell, as shown by the preliminary viability tests. Our experiments highlight the difficulties inherent in attempting to simulate physiological conditions *in vitro*. In order to represent a more natural environment, i*n vitro* flow systems have been used to simulate shear stress on EC (Huang and Eniola-Adefeso, 2012, Pritchard, et al., 1995). Furthermore, insulin failed to stimulate NO production, which may be due to the lower expression of insulin receptor in this cell type compared to macrovascular cells (Gogg, et al., 2009). The microvasculature plays a major role in insulin resistance and represents the largest endothelial surface exposed to circulating lipids (Serne, et al., 1999). The investigation of EF and insulin sensitivity in microvascular EC is thus of great interest to further our understanding of systemic endothelial dysfunction and the resulting impact on diastolic blood pressure. The reduced responsiveness of HDMEC was thus a major limitation in our study, and prevented the observation of the effects of FA profiles on insulin induced NO production. The use of human serum taken from subjects having consumed a high SFA, MUFA, *n*-6 or *n*-3 PUFA meal could have a different impact on EF in HDMEC and other cell types and could be considered as future work.

In our human study, the subcutaneous microvascular bed could be observed by capillaroscopy in the finger, allowing the measurement of capillary density. While capillary rarefaction has been associated to CVD risk factors - particularly hypertension - for more than a decade (Antonios, et al., 1999), intervention studies, and especially dietary interventions, are only at the beginning. The EPA and DHA trial was to our knowledge the first study that looked at the effect of dietary FA on capillary density in humans. We showed that neither EPA nor DHA (~3 g/d, 6 weeks) had an effect on capillary density in young healthy men. Because this was an exploratory outcome, it was not possible to calculate the sample size necessary to observe an effect. However,

the means and standard deviations observed in our study suggest that it was likely to have beenunder-powered for those outcomes. It is worth noting that animal studies have shown a beneficial effect of fish oils on functional capillary density in the ventricles of hypertensive rats (Mitasikova, et al., 2008), and in the cheek pouch of hamsters (Conde, et al., 2007). In these studies, functional capillary density was defined by the activity of enzymatic markers of capillary network (Mitasikova, et al., 2008) or the number of capillaries with flowing red blood cells (Conde, et al., 2007), rather than just visible capillaries at rest, as assessed in the human finger. In addition to pictures, videos of the same area of the finger could give us more information on the dynamic structure of the capillary network. Capillary density may also be measured next to the nail fold where the small vessels are parallel to the skin (Haenggi, et al., 1995). In this area, the structure (tortuousness, diameter) of capillaries may be analysed, as well as the velocity of the red blood cells by imaging techniques (frame to frame, flying spot, cross correlation) (Gasser and Buhler, 1992, Mawson and Shore, 1998, Mugii, et al., 2009). The technique the most commonly used however is the laser Doppler imaging. In this technique, the laser beam is reflected by red blood cells moving perpendicular to the skin. The flowing cells give the laser light a Doppler shift (frequency shift) that is directly proportional to their velocity (Briers, 2001, Silverman, et al., 1994). Velocity measurements are technically demanding, require the subject to remain extremely still and a great expertise from the investigator. The analysis of the dynamic microvascular network, beyond capillary density, represents a great insight in the understanding of endothelial and vascular function. Techniques are expanding in this field and should be considered in nutritional interventions.

Microvascular function relates to BP, as well as EF (Cheng, et al., 2008, Cheng, et al., 2008, Conde, et al., 2007). In line with the capillary density measurement, neither BP nor EF, as assessed by EPC and NOx measurements, was affected by the fish oils intervention. EPC were defined as CD34+/KDR+/CD133+ cells (early EPC) and CD34+/KDR+/CD31+ cells (late EPC). One major limitation of the EPC technique is the very low number of cells amongst total mononuclear cells, which limits the reproducibility of the counting. The lack of effect of fish oils on EPC is consistent with recent findings where fish oil supplementation for 12 weeks (4 g/d) failed to improve CD133+/KDR+ in type 2 diabetics (Wong, et al., 2010). EPC count has also been measured by this techniques in our department in healthy subjects (aged 45-70 y) after 12 months with low doses of EPA+DHA (0.45-1.8 g/d) (Sanders, et al., 2011). The EPC

count, as defined by CD133+/KDR+ and CD34+/KDR+ remained unchanged (Maniou Z, 2011) while the counting of early and late EPC – co-expressing 3 markers - is still under analysis. Techniques to increase the EPC count such as immunomagnetic CD34+ cell enrichment (Distler, et al., 2009, Kuwana and Okazaki, 2012) have been developed and may be of use in future research *in vivo*. Alternatively, EPC could be isolated from participants after supplementation with fish oils, and cultured *ex vivo*, allowing the assessment of EPC function and colony forming units count.

Although this was not part of our main outcomes, one effect worth mentioning is the opposite effect of EPA and DHA observed on night heart rate. Because this was only observed at night, and because our study may have been underpowered for this outcome, this result should be taken with caution. However, in a larger trial (*n* 224 healthy men), Grimsgaard *et al*. reported more than 10 years ago that DHA and EPA (4 g/d, 7 weeks) significantly decreased and increased resting HR, respectively (by ~2 bpm) (Grimsgaard, et al., 1998). A similar increase in ambulatory HR (24h, day and night) was later observed after EPA treatment (4 g/d, 6 weeks) which did not reach significance, while it was significantly reduced by DHA (Mori, et al., 1999) This effect may contribute to the detrimental effect of fish oils sometimes observed, as in the DART-2 study, in which myocardial infarction patients had significant increased risk of sudden cardiac death after dietary advice to consume fish oil or oily fish (Burr, 2007). The effect of fish oils on HR is likely to be due to their incorporation in cardiac membrane. It remains uncertain whether this effect is due to changes in EPA, DPA or DHA in the cardiac membrane, and this is not easily observable in humans. Because HR is a risk factor for CVD, supplementation with EPA alone in the long term, especially in patients at risk such as those with angina or arrhythmia, should be considered with extra care pending confirmation of the current results in future studies. Accordingly, Mozaffarian *et al* recently recommended the use of a combination of both EPA and DHA, because of their potential complementary effect and the limited literature on the individual impact of each FA (Mozaffarian and Wu, 2012). Further studies on the separate effects of EPA and DHA could be carried out in order to determine the dose effect of their opposing effects on heart rate, together with ambulatory recordings of heart rate variability to derive parameters of sympathetic and parasympathetic regulation of fluctuations in heart rate during the day and night. Finally, we showed that the dynamic of incorporation and conversion between EPA, DHA and DPA may vary depending on the lipid fraction. EPA appeared to be elongated to DHA in NEFA but not

erythrocytes. In contrast the retroconversion from DHA to EPA seemed limited in NEFA compared to erythrocytes. This differential incorporation and retroconversion may also depend on the cell and/or organ considered, as well as individual variability. Examining this dynamic could help understand the differential effect of EPA and DHA on CVD risk factors, and for example on HR.

In conclusion, numerous studies have demonstrated the cardioprotective effects of fish oils in humans, likely to be mediated by their hypotriglyceridaemic, hypotensive, anti-arrhythmic and anti-thrombotic properties depending on levels of intake. Recent data suggest that fish oils also improve arterial stiffness and EF (De Berrazueta, et al., 2009, Rizza, et al., 2009), but the work reported here together with a few other published studies suggest that this is more likely to occur in at-risk populations (Sanders, et al., 2011), such as those with pre-existing vascular disease or diabetics. Most studies have investigated the effect of oily fish or fish oil supplements containing mixtures of EPA and DHA, and current UK dietary guidelines recommend the consumption of two portions of fish, one of which should be oily, a week to maintain general good health. However over the past 20 years, there has been growing evidence that EPA and DHA exert a heterogeneous effect on various CV outcomes, which is of considerable relevance for primary and secondary CV prevention. The research presented in this thesis corroborates this to an extent in that EPA increased night-time heart rate whereas DHA decreased it in young healthy men. The *in vitro* endothelial cell experiments also provide support for the theory that circulating NEFA composition may impact on EF.

The apparent efficacy of DHA in improving a number of CVD risk factors, and the remaining uncertainty surrounding the actions of EPA, suggests that the production of *n*-3 PUFA oils that are a purified or enriched source of either EPA or DHA is an important development in the nutraceuticals industry. An increasing number of studies are being published on the cardioprotective effects of DHA triacylglycerol from algal sources, either *Crypthecodinium cohnii* or *Schizochytrium* sp. (Martek Biosciences Corporation, Columbia, MD, USA). Supplements, infant formula, infant foods, and certain other food categories fortified with algal DHA are now available to buy in many countries, with potential benefits for subgroups who have low intakes, such as vegetarians. EPA triacylglycerol-enriched oils and purified EPA ethyl ester oils are

available but effort is also being directed towards development of non-fish oil-derived EPA. As more DHA- and EPA-only products become available, partly as a result of concern over the sustainability of fish oil supplies and partly in response to consumer demand for non-fish sources, future research can be focused on establishing the most effective doses of DHA and EPA for improvement of CVD risk factors. This will inform dietary advice on the optimal intake for life-long health, and should enable a decision to be made on the most effective supplement dose to be taken over short periods to reduce risk factors such as hypertriglyceridaemia or hypertension in various at-risk populations. It will be important to bear in mind that not all individuals will respond to DHA and/or EPA in the same way, and ongoing nutrigenetic and gender research will be crucial in defining future advice regarding dietary and supplementary EPA and DHA. The role of dietary *n*-3 PUFA in cardiovascular health is an area of nutritional science/medicine that has undergone more investigation than most during the past 30 or more years, yet the gaps in our understanding of this field remain substantial.

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Appendix I **KCL RECRUITMENT EMAIL CIRCULAR**

"Circular e-mail for use for recruitment of volunteers for study ref. 08/H0805/2, approved by Bromley Research Ethics Committee. This project contributes to the College's role in conducting research, and teaching research methods. You are under no obligation to reply to this email, however if you choose to, participation in this research is voluntary and you may withdraw at any time"

INVESTIGATION INTO INCORPORATION OF (N–3) POLYUNSATURATED FATTY ACIDS, EICOSAPENTAENOIC ACID AND DOCOSAHEXAENOIC ACID, INTO ERYTHROCYTE MEMBRANES AND EFFECTS ON NOVEL MARKERS OF CARDIOVASCULAR RISK

Short title: The EPA and DHA Trial

We need you to help us investigate how different omega-3 fatty acids (eicosapentaenoic acid, abbreviated to EPA, and docosahexaenoic acid, abbreviated to DHA) influence circulating levels of factors in the blood that are related to vascular health and the elasticity of your arteries.

If you are a healthy male, non-smoker and are aged between 18 and 45 years, and not receiving any medication for blood pressure or blood cholesterol then you may be able to help us.

By taking part in this research you will get information about your cardiovascular risk (computed from your age, blood fats, cholesterol level, blood pressure, body mass index) and haemoglobin level.

All you need to do is:

- Attend the Metabolic Unit in the Department of Nutrition and Dietetics (4th Floor Franklin-Wilkins Building, King's College London) for a screening visit; we will ask you questions about your medical history and dietary habits, measure your height, weight, percentage body fat, waist and hip circumference and blood pressure. We will also take a small blood sample (16.5 ml / ~3 teaspoons) to assess your suitability for the study. Breakfast will be provided afterwards.

- Should you be suitable to take part, you would be asked to take oil capsules (5 per day) for 2 weeks and then attend the Metabolic Unit in the Department of Nutrition & Dietetics for a total of 2 occasions, 6 weeks apart, still consuming 5 oil capsules each day. Therefore you will be consuming 5 capsules per day (which either contain olive oil, fish oil rich in EPA or fish oil rich in DHA) for a total of 8 weeks. We shall ask you to avoid any strenuous exercise on the day before each of the main study visits and to avoid foods high in fat. We shall also ask you to fast overnight, to collect a urine sample for 24 h, and to be fitted with a blood pressure monitor which measures your blood pressure for 24 h.

The following morning, we shall weigh you and estimate your body fat content. After resting for 15 minutes, we will measure your blood pressure and your pulse with external probes on your wrist and finger, and measure the small blood vessels in your finger using a special microscope camera. Following this we shall collect a small blood sample (47 ml / ~9 teaspoons) from a vein in your arm to measure changes in blood fats and other circulating markers of inflammation and blood vessel tone. Each visit will take 2-3 h and we shall take 111 ml of blood in total over the course of the study (including the screening visit) – this is equivalent to about 22 teaspoons of blood. We shall provide you with capsules to consume for two weeks before your first study visit (after the initial screening visit), and then you will be provided with more capsules to consume every day for the next 6 weeks until your final study visit.

The risk associated with the blood collection is small, but there may be a small amount of bruising.

You will be compensated for your time on completion of the study.

The study has been approved by the Bromley National Research Ethics Committee

(Reference No. 08/H0805/2) and will take place from May 2009 to September 2009.

Thank you for your interest.

For further information please contact:

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Aseel Al Saleh aseel.alsaleh@kcl.ac.uk
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Appendix II **ADVERTISEMENT OF THE STUDY**

KING'S College LONDON
University of London

VOLUNTEERS

WANTED

FOR NUTRITION RESEARCH

We are running a study to look at the effects of omega-3 fish oil capsules on your health.

These oils are of great interest as a higher dietary intake of them has been shown to decrease the risk of fatal coronary heart disease and stroke.

We are looking for men:

- Aged 18-45 years
- Willing to take part in an 8-week nutrition study
- Able to attend King's College London, near Waterloo Station for 2 visits of 2h spread throughout the study

We will provide you with oil capsules for the duration of the study

You will be reimbursed for participation in the study and travel expenses

Please contact Sarah Cottin on 0207 848 4594 or e-mail sarah.cottin@kcl.ac.uk

Appendix III INFORMATION SHEET FOR PARTICIPANTS

REC Protocol Number **08/H0805/2**

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

The EPA and DHA trial

We would like to invite you to participate in this original research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this study?

The omega-3 fatty acids found in oily fish are of great interest as a higher dietary intake of these omega-3 fatty acids has been shown to decrease the risk of fatal coronary heart disease (CHD) and stroke. The mechanisms by which omega-3 fatty acids found in oily fish may offer protection are yet to be explained, and it is not known whether the different types of omega-3 fatty acids differ in their effects in the body or whether they act in the same way. The main types of omega-3 fatty acids found in oily fish are ***eicosapentaenoic acid (EPA)*** and ***docosahexaenoic acid (DHA)***. This research project is a randomised controlled trial that compares capsule intakes of different mixtures of omega-3 fatty acids equivalent to 6 portions of oily fish each week on measurements that are related to cardiovascular disease risk. Study participants will be aged 18-45 years.

Why have I been chosen?

You have been contacted as you have expressed an interest in our research. In order to participate in this study you need to be able to say 'Yes' to the following:

- I am male
- I am aged between 18 and 45 years
- I do not smoke
- I have never had a heart attack, stroke, high blood pressure (>160/90mm Hg), liver diseases, diabetes, chronic gastrointestinal disorder or cancer
- I do not have a history of excess alcohol intake or substance abuse
- I am prepared to take 5 capsules a day for 8 weeks

What will happen to me if I take part?

If you would like to participate you would first need to complete a screening questionnaire with us over the telephone (approx. 10 mins), after which potentially eligible volunteers will be invited to attend a clinic

screening appointment (approx. 45 mins) in the Metabolic Unit on 4th Floor, Corridor B, Franklin-Wilkins Building, Stamford Street, SE1 9NH. Volunteers will need to attend this visit after an overnight fast.

The study will be explained in detail and you will be able to ask any questions you may have to ensure you will be giving fully informed consent. Following the signing of the consent form, your height, weight, waist/hip circumference and percentage body fat measurements will be recorded, and a fasting blood sample (approx 16.5mls/ ~3 teaspoons) will be taken to determine whether liver function, haematology, blood glucose and blood lipids are within normal ranges. Seated blood pressure will be measured using an automated sphygmomanometer (blood pressure monitor) that conforms to the recommendation of British Hypertension Society. Refreshments will be provided once all samples and measurements have been made.

Eligible subjects will be asked to complete an 8-week period taking 5g oil each day in capsules (either 10 x 500mg or 5 x 1g) whilst following dietary advice to avoid oily fish intake (e.g. salmon, herring, fresh tuna, mackerel, trout, or sardines) as well as fish oil/cod liver oil supplements. You can still eat as much white fish (e.g. cod, plaice, hake, etc), tinned tuna and shellfish as you choose. We will give you a detailed list of types of fish to avoid. You will be randomly allocated to 1 of 3 groups: 1 group will take olive oil capsules, 1 group will take fish oil capsules which contain more EPA than DHA, and the other group will take fish oil capsules which contain more DHA than EPA. You will have an equal chance of being allocated to any one of these groups.

During the intervention, you will be asked to attend the Metabolic Unit at the Franklin-Wilkins Building twice, after 2 weeks and in the end of the intervention. The week before each visit, you will be given urine bottles and an ambulatory blood pressure monitor to use 2 or 3 days before your next study visit; and we will explain in detail how to collect your urine for 24 h and how to use the blood pressure monitor.

At the end of the 2-week period you will attend the Metabolic Unit at the Franklin-Wilkins Building for your first study visit, bringing with you the collected urine and blood pressure monitor which you will have used over 24 h. We will make measurements to assess the functioning of the large and small blood vessels in your wrist and finger and take a blood sample (approx. 47 ml/ ~9 teaspoons. This visit lasts 2 to 3 h and will be repeated at the end of the intervention period. See figure 1 for an outline of these visits. To standardise everyone before these visits you will be asked to avoid strenuous physical activity, foods high in fat, caffeine or alcohol on the day before the visit. Only water will be allowed for 12 h before the scheduled study visit.

The overall study is shown in a diagram below:

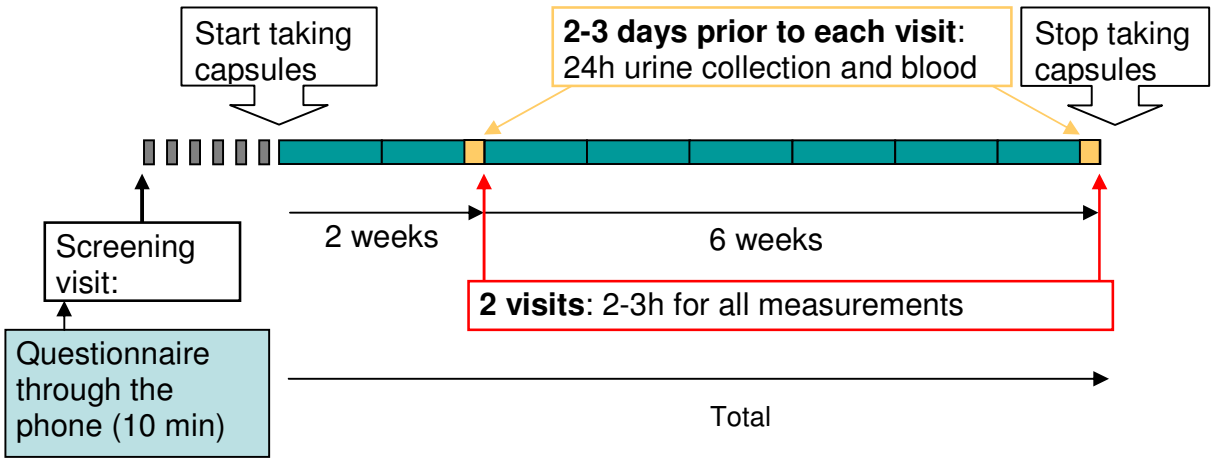


Figure 1. Study outline.

Screening visit:

1) **You should avoid eating or drinking anything, except water, for 12 h before your scheduled screening visit.**
2) **The visit will last approximately 45 min.**
3) **We will give you a copy of this information sheet, explain to you all the details of the study and answer any questions you have. If you are still happy to take part in the study, you will be asked to sign a consent form.**
4) **We shall ask you questions about your medical history, your food habits through a Food Frequency Questionnaire and measure your weight, height, percentage body fat, blood pressure and waist and hip circumference.**
5) **We will need to take a small blood sample (16.5 ml /~ 3 teaspoons) to check that your blood chemistry is normal.**
6) **Then you will be provided with breakfast.**
7) **The results of the screening blood test will be given to you. If any abnormal results are found we will inform you immediately and we will provide you with a letter for your GP, which we will advise you to give to your GP.**

Main study:

1) **Following screening, if your results comply with the study inclusion criteria you will be invited to attend the Metabolic Unit in the Franklin-Wilkins Building on 2 further occasions; each of these visits will take approximately 2-3 h each.**
2) **We shall ask you to avoid eating oily fish and consuming fish oil supplements for 2 weeks prior to the start of the study and during the study. We will provide you with study capsules containing a test oil and ask you to consume 5 of these capsules per day. We will also ask you to avoid fatty foods, drinking alcohol, and any strenuous exercise the day prior to each visit to the Department of Nutrition & Dietetics.**
3) **We shall also ask you not to consume caffeine from midday the day before each visit and to avoid eating or drinking anything, except water, for 12 h before your scheduled study visit.**
4) **2 to 3 days prior to each of the 2 remaining visits, you will be asked to collect urine samples and to record your blood pressure (using the ambulatory blood pressure monitor) for 24 h.**
5) **You will be asked to report to the Metabolic Unit in the Department of Nutrition & Dietetics between 08:00 h and 11:00 h, in the fasted state (i.e. without having consumed breakfast and without having consumed any food or drink for 12 h, apart from water). Make sure you drink some water on the morning of the study to avoid dehydration.**
6) **At each of the 2 visits (at 2 wk and 8 wk), we will then measure your blood pressure and the function of your large and small blood vessels. We shall make measurements of blood pressure using a sphygmomanometer, in which a cuff will be placed around your arm and will be inflated. The cuff causes a tingling sensation in your arm, but does not cause pain. The measurements we make to assess the function of your larger blood vessels are carried out using probes that are gently placed on your wrist and finger to determine the elasticity or tone of your arteries. The measurement of small blood vessel function involves you placing the top of your finger under a camera which can image individual capillaries (the smallest type of blood vessel) to monitor the capillary blood flow before and after inflation of a cuff placed around your forearm. All of these measurements are non-invasive and will not cause any discomfort. In the end of the visit, we will take a small sample of blood: 47 ml /~9 teaspoons.**
7) **Finally, following these measurements you will be offered refreshments.**

After the second visit (at 2 wk), you will be provided with more oil capsules for the remainder of the study and you will continue to take 5 capsules per day for 6 weeks. You will be advised to avoid oily fish and to abstain from taking fish oil supplements or cod liver oil for the duration of the study (8 weeks in total).

Will my participation be kept confidential?

Any information collected about you during this research will be kept strictly confidential. Your GP will not be told that you are taking part in the study, unless you request us to do so. Subject confidentiality and anonymity will be observed throughout the study by use of subject codes in place of names, and the storage of subject details in a secure place. Only the investigators have access to this data. Should you wish to find out the results of this study you are welcome to contact Dr Wendy Hall (details below) for a copy of the final report once the study is finished.

What will happen to my study results?

We hope to publish the results of the whole study in a scientific journal. You will not be identified in any publication. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who is organising and funding the study?

The study is organised and funded by the Nutritional Sciences Division, Kings College London. In recognition of your time commitment, you will be paid an honorarium of £50 upon completion of the study.

Do I have to take part?

It is up to you to decide whether to take part or not. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you decide to take part, please let us know if you have been involved in any other study in the last year.

If this study has harmed you in any way you can contact King's College London using the details below for further advice and information:
Thank you for your interest.

For further information, please contact:

Sarah Cottin, email: sarah.cottin@kcl.ac.uk
or **Aseel Al Saleh, email: aseel.a.alsaleh@kcl.ac.uk**

Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London, SE1 9NH

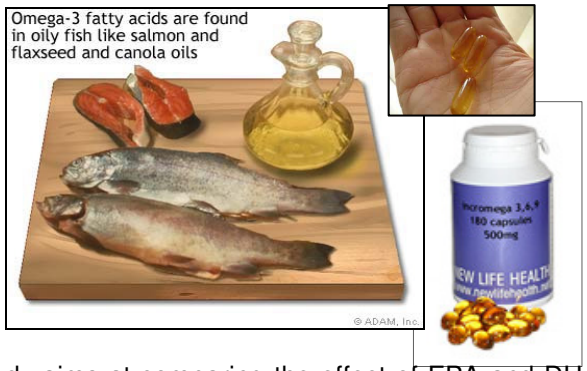
Chief Investigator: Dr Wendy Hall Tel. 020 7848 4197 wendy.hall@kcl.ac.uk

Co-investigators: Prof Tom Sanders Tel. 020 7848 4273 tom.sanders@kcl.ac.uk; Dr Zoe Maniou Tel. 020 7848 4546 zoitsa.maniou@kcl.ac.uk

Appendix IV **STUDY INFORMATION BOOKLET**

The EPA and DHA trial

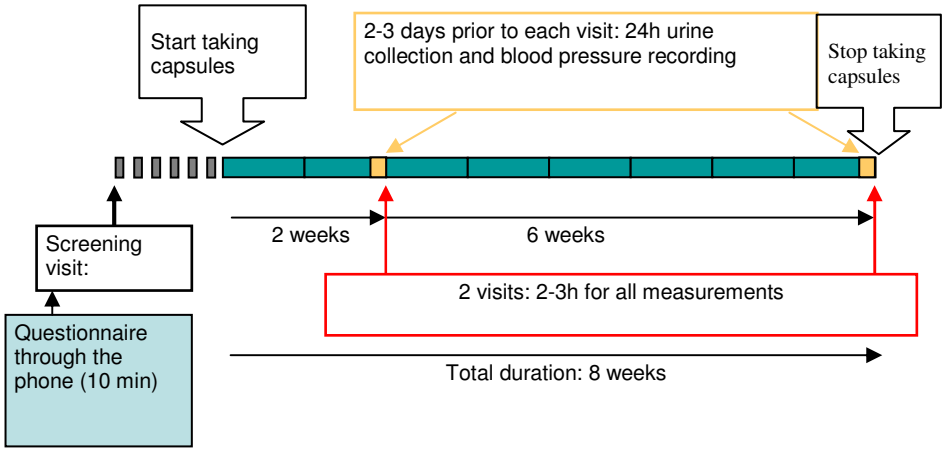
A high intake of fish oils, as observed in the Inuit population, is associated to a reduced risk of heart diseases and insulin resistance. Fish oils are composed of two types of omega-3 fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The beneficial effects of fish oils have been well established and omega 3 supplements have flourished on the market over the past decade. However, it remains unclear if EPA, DHA or a combination of both are required to improve insulin sensitivity and cardiovascular health.



This study aims at comparing the effect of EPA and DHA on different biological processes involved in the development of cardiovascular diseases, including inflammation, thrombosis and endothelial function.

STUDY OUTLINE

The nutritional intervention (outlined below) lasts 8 weeks during which you will be asked to take 5 capsules a day containing one type of fat (either EPA, DHA or olive oil as a placebo). You will be required to attend the Metabolic Unit at the Franklin-Wilkins Building, Waterloo Campus, of King's College London on 3 different occasions: one before (screening) and 2 during the intervention.



THE DAY BEFORE THE VISITS

Prior to each of the 3 visits (screening or intervention) you should avoid having caffeine from midday, and also avoid doing exercise and drinking alcohol for 24h. You will be asked to have a low fat meal in the evening (instructions will be given) fast overnight (no food or drink, except water, from 10pm, and no breakfast on the study day). You will then attend the Metabolic Unit at a pre-arranged time between 8.30 and 10.30 am. It is important that you drink water before attending.

FIRST VISIT: SCREENING (45 min)

The purpose of this screening session is to determine whether you are suitable to participate in our study. This involves measurement of your height, weight, waist circumference, body fat and blood pressure. It also involves the collection of a blood sample to determine your blood fats and glucose levels. We will give you more information and answer any question you might have regarding the study. Breakfast will be provided In the end of the screening session.

2 VISITS DURING THE INTERVENTION (2-3H)

You will be required to attend the Metabolic Unit at the Franklin-Wilkins Building, Waterloo Campus, of King's College London after 2 weeks and in the end of the intervention (8 weeks).
2 or 3 days prior to each visit, you will be asked to collect your urine and to record your blood pressure for 24h: A cuff will be put on your

left arm and inflate every half an hour during the day and every hour at night.
Each visit will consist in non invasive vascular measurement (sensors will be placed on your finger and your wrist) and blood taking. Lunch will be provided in the end of each visit.

FISH OILS RESTRICTION

We shall ask you to avoid eating oily fish, such as salmon, trout or mackerel, and consuming fish oil supplements for the 8 week intervention period. However, you will still be allowed to eat non oily fish such as cod, haddock or sea bass. A more detailed list of fishes allowed/to avoid will be provided.

STUDY DATES

Please choose the date you will start the intervention, taking into account your availability for the 2 following visits.

	Date	Time
Day starting taking capsules (time 0)		-
2 week point		
8 week point		

WHAT YOU WILL GET BY TAKING PART

- Information on your blood fats, glucose levels and full blood count
  - A full biochemical screening
- Information on your body composition, physical measurements, vascular health and blood pressure
  - £50 for your time

By participating in our study you will provide us with valuable information. Your commitment to our study is greatly appreciated.

Thank you!

CONTACT DETAILS:

Aseel Alsaleh aseel.alsaleh@kcl.ac.uk,

Sarah Cottin sarah.cottin@kcl.ac.uk,

020 7848 4594

Appendix V **HEALTH QUESTIONNAIRE**

The EPA and DHA Trial

Please complete this questionnaire and return by email to
sarah.cottin@kcl.ac.uk **or** *aseel.alsaleh@kcl.ac.uk*,
School of Biomedical and Health Sciences, King's College London,
Franklin-Wilkins Building Room 4.46A, 150 Stamford Street
London SE1 9NN

* Please delete as applicable

NAME:

ADDRESS:

AGE:

D.O.B:

GENDER:

HEIGHT:

WEIGHT:

TELEPHONE NUMBER:

Email:

GP Name and Address:

Have you recently taken part in any other trial? YES / NO*

Do you smoke? YES / NO*

Do you drink alcohol? YES / NO*

If so, how many units per /week?
1 unit = glass of wine, shot of spirits,
1/2 pint of beer

……. Units day / week*

Do you suffer from any allergies? YES / NO*

If so please give details:

Do you suffer from any medical problems? YES / NO*

If so please describe:

Are you taking any medication? YES / NO*

If so please give details:

Do you take any dietary supplements? YES / NO*

How frequently do you consume oily fish? >1 portion per wk/1 portion per wk/<1 portion per wk
(explain what oily fish are)

Do you have a history of any of the following?

High blood pressure YES / NO*

Diabetes mellitus YES / NO*

Myocardial infarction-heart attack/angina/stroke/thrombosis YES / NO*

Cancer YES / NO*

Liver or gastrointestinal disorders YES / NO*

Are you receiving medication for any of the following?

Raised blood cholesterol YES / NO*

High blood pressure YES / NO*

Immune system (anti-histamines, anti-inflammatory drugs) YES / NO*

Do you have any special dietary requirements?

Lacto-ovo vegetarian/vegan/other (specify) YES / NO*

Religious dietary requirements (specify) YES / NO*

Other YES / NO*

Appendix VI **OILY FISH RESTRICTION**

Restriction in oily fish consumption

You are required to avoid any source of fish oils, including supplements or oily fishes (left of the table below) during the 8 week intervention period

Oily / fatty fish to avoid	White / non-oily fish allowed
Salmon	Cod
Trout	Haddock
Mackerel	Plaice
Herring	Coley
Sardines	Whiting
Pilchards	Lemon sole
Kipper	Skate
Eel	Halibut
Whitebait	Rock Salmon/Dogfish
Tuna (fresh only)	Ayr
Anchovies	Catfish
Swordfish	Dover sole
Bloater	Flounder
Cacha	Flying fish
Carp	Hake
Hilsa	Hoki
Jack fish	John Dory
Katla	Kalabasu
Orange roughy	Ling
Pangas	Monkfish
Sprats	Parrot fish
	Pollack
	Pomfret
	Red and grey mullet
	Red fish
	Red Snapper
	Rohu
	Sea bass
	Sea bream
	Shark
	Tilapia
	Turbot
	Tinned tuna
	Marlin

Ref: http://www.food.gov.uk/news/newsarchive/2004/jun/oilyfishdefinition

Appendix VII FATTY ACID ANALYSIS (GAS CHROMATOGRAPHY) OF EPA- AND DHA- RICH OILS

VII. A. TYPICAL FATTY ACID COMPOSITION PROVIDED BY THE MANUFACTURER (INCROMEGA EPA500TG SR AND DHA500TG SR)

	EPA-rich capsules	DHA-rich capsules
Fatty Acid	Content (%)	Content (%)
C14:0	0.5	-
C16:0	0.9	0.7
C16:1	0.7	0.3
C16:2	0.7	0.5
C16:3	0.5	0.1
C16:4	0.5	-
C18:0	0.4	3.7
C18:1	3.7	6.9
C18:2	2.1	1.6
C18:3 n-6	0.6	0.5
C18:3 n-3	1.1	0.2
C18:4 n-3	5.6	0.3
C20:0	0.5	0.6
C20:1	0.2	2.2
C20:2 n-6	0.8	0.5
C20:3 n-6	0.6	0.2
C20:4 n-6	4.1	2.8
C20:4 n-3	3.2	0.7
C22:0	-	0.3
C20:5 n-3	**58.4**	**8.3**
C22:1	1.7	0.9
C21:5 n-3	1.6	0.5
C24:1	-	0.6
C22:5 n-6	0.4	4.6
C24:0	-	0.4
C22:5 n-3	1.5	3.1
C24:1	-	1.3
C22:6 n-3	**8.8**	**57.5**
Others	0.9	0.7
Total *n*-3 PUFA	**67.2**	**65.8**
Total VLC *n*-3 PUFA	68.7	68.9
Total *n*-6 PUFA	6.5	8.6
Total MUFA	6.3	12.2
Total SFA	2.3	5.7

VII. B. FATTY ACID COMPOSITION MEASURED IN OLIVE OIL, DHA- AND EPA-RICH CAPSULES

	Olive oil capsules	DHA-rich capsules	EPA-rich capsules
Fatty Acid	Content (%)	Content (%)	Content (%)
16:0	10.71	0.96	0.00
16:1	0.75	0.00	0.93
18:0	3.42	3.51	1.82
18:1n-9	**76.62**	**6.27**	**0.78**
18:1n-7	2.06	1.23	2.51
18:2n-6	4.69	1.16	0.66
20:5n-3	**0.00**	**10.34**	**61.97**
22:4n-6	0.00	4.25	1.80
22:5n-6	0.00	1.27	0.00
22:5n-3	**0.00**	**3.34**	**2.06**
22:6n-3	**0.00**	**57.31**	**14.10**
Total VLC *n*-3 PUFA	0.00	70.98	78.13
Total *n*-6 PUFA	4.69	6.68	2.46
Total MUFA	79.43	7.50	4.22
Total SFA	14.13	4.47	1.82

PUFA, Polyunsaturated fatty acids; VLC *n*-3 PUFA, very long chain *n*-3 PUFA (20:5n-3 + 22:5n-3 + 22:6n-3); MUFA, Monounsaturated fatty acids; SFA, saturated fatty acids

Appendix VIII **CONSENT FORM**

CONSENT FORM FOR PARTICIPANTS IN A NUTRITIONAL STUDY

- Please complete this form after you have read the Information Sheet and you are satisfied that the research has been fully explained.
- Title of Study: The EPA and DHA Trial
- Research Ethics Committee Ref: 08/H0805/2

Thank you for considering taking part in this research. The person organizing the research must explain the project to you before you agree to take part.

If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to participate. You will be given a copy of this Consent Form to keep and refer to at any time.

- *I confirm that I fit into the following criteria*

  - *I am a male aged between 18 and 45 years and do not smoke*
  - *I do not have a history of heart disease, stroke, high blood pressure, diabetes, thrombosis, liver disease, chronic gastrointestinal disorders or a cancer diagnosis (except basal cell carcinoma)*
  - *I do not have a history of excess alcohol intake or substance abuse*

- *I understand that if I decide at any other time during the research that I no longer wish to participate in this project, I can notify the researchers involved and be withdrawn from it immediately.*
- *I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.*
- *I agree that the research team may use my data for future research and understand that any such use of identifiable data would be reviewed and approved by a research ethics committee. Please note that in such cases, as with this project, confidentiality and anonymity will be maintained ant it will not be possible to identify you from any publication*

Participant's Statement:

I _____

agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves.

Signed _____ Date _____

- Investigator's Statement:

I _____

confirm that I have carefully explained the nature, demands and any foreseeable risks (where applicable) of the proposed research to the volunteer.

Signed _____ Date _____

Appendix IX STANDARD PHYSICAL MEASUREMENT PROCEDURES

1. Height

Ensure that subject has removed all ornamentation from his/her hair, and is barefoot. Using a wall mounted stadiometer, ensure that the volunteer stands upright with heels and shoulders against the measuring rod, knees and back straightened, and looking straight ahead. Lower the measuring slide onto the participants head, so that it compresses the participants' hair. The measuring slide should not bend. Record the participants' height at the 'read-off' mark.

2. Waist circumference

Waist circumference should be measured over light clothing, so that the tape measure can be held as closely against the body as possible. The measurement should be read to the nearest 0.1cm. The subject should be standing upright when the measurement is taken, and the measurement should be taken at the minimum circumference between the rib cage and the iliac crest.

3. Body composition by bio-electrical impedance

Body composition (and weight) should be assessed using a Tanita BC-418 segmental body composition analyzer. After turning on the power, all the required data should be entered. Standard settings should be used for clothes weight (1.0 kg) and body type (normal, not athletic). Enter the information about the participants' gender, age and height, and then wait for the flashing arrow to appear next to the 'step on' prompt, before asking the subject to step onto the scales platform. Ask the participant to step onto the weighing platform with bare feet and to take a handgrip firmly into each hand, and allowing each arm to hang straight down along their sides without touching their body.

The display will emit short beeping sounds while the body composition measurements are being determined. When the measurements are complete, a detailed summary of the results will be printed. The display will then automatically return to zero. Attach the print out to the screening patient record being used to manually record the other measurements being taken at the same screening visit.

4. Blood pressure and heart rate

Blood pressure should be taken using an Omron 705IT auto upper arm blood pressure monitor. When taking a blood pressure measurement, the volunteer must be calm and relaxed. Participants should be allowed to rest quietly at a comfortable room temperature for five minutes before the measurements are performed. Participants

should not be drinking, eating, smoking or talking at the time that the blood pressure will be measured.

Ensure that the subject is sitting quietly with their legs uncrossed and feet flat on the floor. The arm that the measurement is being taken from needs to be comfortably supported to heart level (arm below this tends to underestimate both systolic and diastolic blood pressure by approximately 10 mmHg, and above the heart tends to overestimate). Select the appropriately sized cuff and place the bladder of the cuff 2-3 cm above the antecubital fossa in the brachial artery (around the participants' upper arm). Wrap the cuff snuggly around the arm, making sure that the cuff index line falls within the marked ranges. There should be space for a finger between the participants' arm and the cuff. The cuff must be placed so that the participants' artery is aligned with the cuff arrow marked 'artery'.

Press the start/stop button and allow the reading to take place. It takes about 40 seconds for a measurement to be completed. If a reading is not detected, the cuff will automatically re-inflate. In some instances, the reading will fail yet again. This may be due to the fact that the cuff is not aligned correctly. It is thus important to ensure from the start that the cuff arrow is correctly aligned with the artery (feel for a pulse to be certain).

Document the readings for systolic and diastolic blood pressure, as well as the heart rate reading. Repeat the measurements of blood pressure two more times at two - five minute intervals, and discard the first measurement.

Appendix X **KCL SCREENING RECORD SHEET**

Date		
Name		
Code (for screening)		
Date of birth		
Consent form signed?	YES / NO	
Health questionnaire received?	YES / NO	
Is participant satisfied with the restriction in oily fish intake?	YES / NO	
Blood pressure (mmHg) To be taken at 2-5 minute intervals	**Reading 1: (S)** **(D)** **Reading 2: (S)** **(D)** **Reading 3: (S)** **(D)** **Average of reading 2 & 3: (S)** **(D)**	**Pulse:** **Pulse:** **Pulse:** **Pulse:**
Height (m)		
Body composition taken?	YES / NO (attach copy)	
Weight (kg)		
Waist circumference (cm)		
Hip circumference (cm)		
Blood samples taken?	YES / NO 4ml (FX), 4ml (EDTA), 8.5 ml (serum)	
Intervention period	From ……/…… to ……/……	

First visit (2 weeks)

Second visit (8 weeks)

Phlebotomist

Phlebotomist's comments	
Telephone number	
Notes:	

Appendix XI **BLOOD HANDLING PROTOCOL (SCREENING)**

All analysis was done at King's College Hospital (KCH) under the supervision of Dr Roy Sherwood

SAMPLE	TUBES	ICE/RT	CENTRIFUGE	SEPARATION	ANALYTES	STORAGE	TRANSPORT TO KCH
Fasting ***(16.5 ml)***	Fluoride oxalate (grey) (1 * 4 ml C/P 368587)	Ice	15 min x 1300 g @ 4ºC	Separate plasma into 2 x 1 ml aliquots	Glucose	Freeze 1 aliquot and send the other fresh to KCH	Same day at room temperature
	4 ml EDTA (lavender) (1 * 4 ml C/P 367844)	Room temperature	No	None	FBC	Room temperature	Same day at room temperature
	No anticoagulant (red) (1 * 8.5 ml H/P 367814)	Ice	15 min x 1300 g @ 4ºC	Separate into 2 x 1 ml aliquots	FLIP LFT	Freeze 1 aliquot and send the other fresh to KCH	Same day at room temperature

FBC; full blood counts, FLIP; full lipid count (TAG, total, HDL and LDL cholesterol), LFT; liver function

Appendix XII **TRIGLYCERIDES (TRIG)**

XII. A. EXPLANATION OF THE TEST

Triglyceride concentrations (in conjunction with serum cholesterol) are useful in the diagnosis and classification of the dyslipoproteinaemias (primary and secondary) and in the calculation of LDL cholesterol via the Friedwald formula given the total and HDL cholesterol. They are also used in the diagnosis and treatment of patients with suspected atherosclerosis, poorly controlled diabetes mellitus, nephritic syndrome, liver disease or obstruction and other diseases involving lipid metabolism.

XII. B. METHOD

Triglyceride reagents supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD

The Siemens Advia method for the measurement of triglycerides is an enzymatic assay. Triglycerides are converted to glycerol and free fatty acids by lipase. The glycerol is then converted to glycerol-3-phosphate by glycerol kinase followed by its conversion by glycerol-3-phosphate-oxidase to hydrogen peroxide. A coloured complex is formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The absorbance of the complex is measured as an endpoint reaction at 505/694 nm.

XII. C. TECHNICAL DATA

- *INTRA-ASSAY PRECISION*

	Level 1	Level 2
Mean (mmol/L)	1.32	2.36
N	20	20
SD	0.007	0.011
CV%	0.6	0.5

- *INTER-ASSAY PRECISION*

	Level 1	Level 2
Mean (mmol/L)	1.32	2.36
N	20	20
SD	0.033	0.035
CV%	2.5	1.5

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses

2 µL of sample in the test, although the minimum volume required in the specimen container is 50µL (micro sample cups) or 200µL (Vacutainers and inserts).

- *LINEARITY*

Linear to approximately 10 mmol/L.

- *SENSITIVITY*

The minimum detectable concentration of triglyceride is 0.01 mmol/L.

- *STANDARDIZATION*

The ADVIA triglyceride method measures total glycerols and is traceable to a reference method, which uses reference materials from the National Institute of Standards and Technology (NIST), via patient sample correlation. Assigned values of Bayer Chemistry Calibrator and Bayer Assayed Chemistry Controls are traceable to this standardization.

- *REFERENCE RANGE*

< 2.0 mmol/L (fasting).

Appendix XIII **CHOLESTEROL (CHOL)**

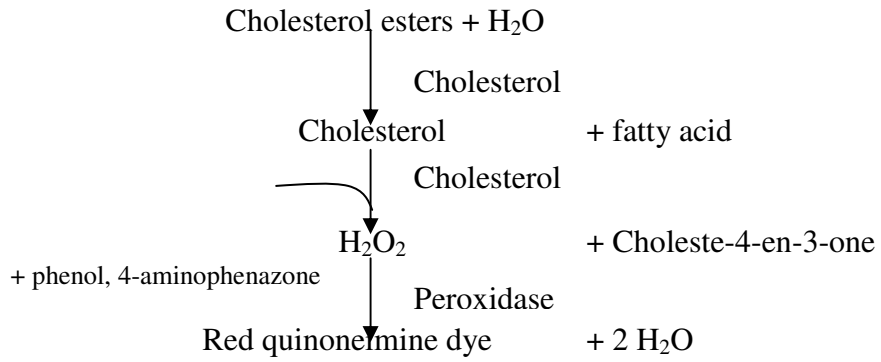
XIII. A. EXPLANATION OF THE TEST

A raised serum cholesterol has been associated for many years with an increased risk of atherogenic plaque formation leading to an increased likelihood of ischaemic heart disease, myocardial infarction and stroke (cerebrovascular accident). At a cholesterol concentration above 5.2 mmol/l (according to the WHO) the risk of atherogenesis rises in proportion to cholesterol concentration and is additive to other risk factors such as smoking, hypertension and family history.

XIII. B. METHOD

Cholesterol reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD

Serum cholesterol is determined using an enzymatic method. Cholesterol esterase completely hydrolyses cholesterol esters in serum to free cholesterol, which is in turn oxidised by cholesterol oxidase generating hydrogen peroxide. The hydrogen peroxide formed combines with 4-aminophenazone and a phenol to form a red quinone amine dye which is measured as an endpoint reaction at 505/694 nm. The increase in dye absorbance is directly proportional to the concentration of cholesterol in the sample, when compared to a previous calibration assay.



XIII. C. TECHNICAL DATA

- *INTRA-ASSAY PRECISION*

	Level 1)	Level 2	Level 3
N	20	20	20

Mean (mmol/L)	3.91	5.15	5.67
SD	0.026	0.034	0.031
CV%	0.6	0.6	0.6

- *INTER-ASSAY PRECISION*

	Level 1	Level 2	Level 3
N	20	20	20
Mean (mmol/L)	3.91	5.15	5.67
SD	0.044	0.080	0.057
CV%	1.1	1.5	1.0

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 2 µl of sample in the test, although the minimum volume required in the specimen container is 50µl (micro sample cups) or 200µl (Vacutainers and inserts).

- *LINEARITY*

Linear to approx. 17 mmol/l.

- *SENSITIVITY*

The minimum detectable concentration of cholesterol is 0.01 mmol/L.

- *REFERENCE RANGE*

Quoting reference values for serum cholesterol in terms of population distribution is actively discouraged in favour of setting desirable targets. Current WHO recommendations suggest a cholesterol concentration below 5.2 mmol/L as being desirable to avoid coronary artery disease.

- *STANDARDIZATION*

The ADVIA cholesterol method is traceable to the CDC reference method, which uses reference materials from the National Institute of Standards and Technology (NIST), via patient sample correlation.

Appendix XIV **HIGH DENSITY LIPOPROTEIN (HDL)**

XIV. A. EXPLANATION OF THE TEST

HDL (high density lipoprotein) is a lipoprotein which plays an essential role in cholesterol transport and metabolism, principally by transporting excess cholesterol from peripheral tissues to the liver. HDL cholesterol measurements enable LDL cholesterol to be calculated (given the total cholesterol and triglycerides), and this is the most important lipoprotein in the assessment of coronary artery disease. HDL itself is also an independent risk factor for such disease being inversely related to the risk.

XIV. B. METHOD

HDL cholesterol reagents supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD.

The Siemens Advia Direct HDL cholesterol method is a two step automated procedure. In the first step cholesterol esterase and cholesterol oxidase react to remove non-HDL cholesterol from the sample. The hydrogen peroxide produced is then removed by the enzyme catalase. The absence of detergent in this first reaction prevents HDL from reacting with the enzymes. In stage 2 detergent is added to allow HDL to react with the enzyme system. Sodium azide inhibits the reaction of the hydrogen peroxide formed with catalase. The hydrogen peroxide acts with 4-aninoantipyrine to produce a quinoneimine pigment measured at 596 nm.

XIV. C. TECHNICAL DATA

- *INTRA-ASSAY PRECISION*

	Level 1	Level 2	Level 3
Mean (mmol/L)	0.91	1.39	1.95
N	20	20	20
SD	0.010	0.019	0.029
CV%	1.1	1.4	1.5

- *INTER-ASSAY PRECISION*

	Level 1	Level 2	Level 3
Mean (mmol/L)	0.91	1.39	1.95
N	20	20	20
SD	0.020	0.029	0.049

CV%	2.2	2.1	2.5

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 2 µl of sample in the test, although the minimum volume required in the specimen container is 50µl (micro sample cups) or 200µl (Vacutainers and inserts).

- *SENSITIVITY*

The minimum detectable concentration of HDL cholesterol is 0.1 mmol/L.

- *LINEARITY*

Linear from 0.4 - 2.3 mmol/L.

- *REFERENCE RANGE*

Quoting reference values for serum HDL cholesterol in terms of population distribution is actively discouraged in favour of setting desirable targets. Current WHO recommendations suggest a cholesterol concentration above 1.0 mmol/l as being desirable to avoid coronary artery disease. HDL cholesterol is also used in conjunction with total cholesterol and triglycerides to calcualate LDL cholesterol.

- *STANDARDIZATION*

The Advia HDL method is traceable to the NCEP Designated Comparison Method (reference method) via patient sample correlation. Assigned values of ADVIA Chemistry HDL/LDL Cholesterol Calibrator and ADVIA Chemistry Lipid Controls are traceable to this standardization.

Appendix XV **GLUCOSE**

XV. A. EXPLANATION OF THE TEST

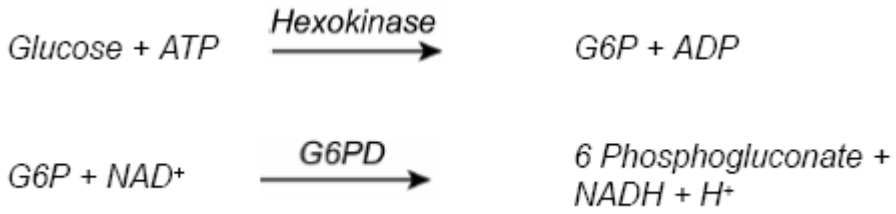
Glucose measurements are used in the diagnosis and management of diabetes mellitus and in the monitoring of patients on parenteral nutrition. Glucose is also of use in calculating osmolarity and in the diagnosis of diseases resulting in hypoglycaemia e.g. insulinoma.

XV. B. METHOD

Glucose reagent is supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD.

Glucose in the sample is phosphorylated by the transfer of phosphate from adenosine triphophosphate (ATP) via the action of hexokinase. Glucose-6-phosphate formed in this reaction is oxidised by glucose-6-phosphate dehydrogenase. This is accompanied by the reduction of NAD+ to NADH which results in an increase in absorbance at 340 nm proportional to the glucose concentration in the sample. The increase in absorbance is converted to glucose concentration by reference to a previously determined calibrator and reagent blank.

The effect of any interfering substances is reduced by blanking each sample using reagent 1 (buffer and co-factors) before the addition of reagent 2 (enzymes).



XV. A. TECHNICAL DATA

- *SAMPLE REQUIREMENTS*

The sample must be taken into a fluoride-oxalate tube as fluoride preserves glucose for at least 48 hours without separation. The plasma may be may be refrigerated overnight or stored at room temperature in seroseparator tubes. CSF and other fluids can

also be analysed with this method - the sample should be collected into a fluoride-oxalate container - if not the sample should be analysed immediately.

The ADVIA 2400 uses 3.4 µl of sample in the test, although the minimum volume required in the specimen container is 50µl

- *INTRA-ASSAY PRECISION*

	Level 1	Level 2
Mean (mmol/L)	4.4	16.3
SD	0.02	0.08
CV (%)	0.6	0.5

- *INTER-ASSAY PRECISION*

	Level 1	Level 2
Mean (mmol/L)	4.4	16.3
SD	0.07	0.19
CV (%)	1.6	1.2

- *LINEARITY*

The assay islinear up to 38.9 mmol/L

- *REFERENCE RANGE*

For fasting samples the reference range is 2.5 to 5.0 mmol/l. WHO guidelines state that a diagnosis of diabetes mellitus can be made on fasting plasma samples with a glucose concentration of 7.0 mmol/L or greater – a single result is acceptable in a symptomatic patient, otherwise two results are required to make the diagnosis. Other WHO guidelines are laid down for the diagnosis and classification of diabetes mellitus which are beyond the scope of this method sheet.

CSF glucose is normally approximately 60% of the current plasma glucose.

No reference ranges are available for other fluids

- *STANDARDIZATION*

The ADVIA glucose oxidase method is traceable to the CDC Reference Method, which uses reference materials from the National Institute of Standards and Technology (NIST), via patient sample correlation and verified with NIST Reference serum. Assigned values of Siemens Chemistry Calibrator, Siemens Assayed Chemistry Controls, and ADVIA Chemistry Urine Controls are traceable to this standardization.

Appendix XVI **FULL BLOOD COUNT (FBC)**

Reagents supplied by Siemens Medical Solutions Diagnostics Limited, Bayer House, Strawberry Hill, Newbery, Berks. RG14 1JA

XVI. A. EXPLANATION OF THE TEST

The Siemens Advia 2120 is used to analyse the full blood count (FBC), Nucleated Red Blood Cell Count (NRBC) and Reticulocyte count. These are important tests requested by the clinician to evaluate the health of a patient. The results are used in conjunction with other diagnostic tests to diagnose, treat and monitor the progress of a patient. The Advia 2120 is a haematology analyser, which has the capacity to analyse the following test profiles: CBC/DIFF, CBC/DIFF/RETIC, CBC/RETIC.

XVI. B. METHOD

The Siemens Advia 2120 uses the following principles of analysis: -

- *HYDRONAMIC FOCUSING*

The sheath/rinse reagent surrounds the sample stream to produce a single stream of cells, which reduces co-incidence during flow cytometry analysis.

- *PEROXIDASE (PEROX) METHOD*

Uses the intracellular myeloperoxidase enzyme to distinguish between the different white cell types. The cells have peroxidase enzyme substrate 4-chloro-1-napthol added in the presence of hydrogen peroxide, which forms a dark precipitate at endogenous sites of peroxidase activity in the granules of the White Blood Cell. The sample is passed through the flowcell and the light scatter, which determines the size of a cell and the absorption, which provides information on the level of staining, is measured. Neutrophils, monocytes and eosinophils are peroxidase positive whereas lymphocytes and basophils are peroxidase negative.

- *BASOPHIL/LOBULARITY (BASO) METHOD*

addition of phthalic acid and a surfactant which lyses the red cells, platelets and the cytoplasm of all white cells except the basophils these pass through the laser and

information collected from the light scatter which corresponds to the nuclear configuration and cell size.

- *CYANIDE FREE HAEMOGLOBIN DETECTION (HGB)*

there are 2 methods employed.

METHOD 1: a cyanide free colourimetric method, which lyses the RBC to release the haemoglobin. The heme ion is oxidised to the feric state where it combines with the reagent to form an axial ligand monoaquomonohydroxyferri-porphyrin. Optical readings are taken at a wavelength of 456nm and plotted on the haemoglobin transmission histogram. This method is affected by background colouration e.g. lipaemia, haemolysis and icteric samples.

METHOD 2: Cellular HGB is a calculated method which uses the directly measured parameter CHCM (Corpuscular Haemoglobin Concentration Mean) it is calculated using the following values CHCM x RBC x MCV /1000. This method is not affected by either haemolysed, lipaemic or icteric samples.

The 2 methods should not differ by more than 1.9g/dl however if they do an error message is flagged. The CHCM should only be used when the haemoglobin is falsely increased and the MCHC is also falsely increased. Using CHCM to calculate Cellular HGB avoids this interference since CHCM is directly measured and is virtually unaffected by lipaemia. To calculate HGB, replace MCHC with CHCM as follows:

MCHC = (HGB ¸ [RBC x MCV]) x 1000

HGB = (MCHC x RBC x MCV) / 1000

Cellular HGB = (CHCM x RBC x MCV) / 1000 or (CHCM x Hct) / 100

- *NRBC COUNT*

Nucleated Red Blood Cells are identified by the nuclear size in the peroxide channel, and by nuclear density in the Basophil/Lobularity channel. Within the unstained region of the peroxidase channel the NRBC nuclei are located between the noise and lymphocytes and form distinct populations (1), which can be analysed to produce a count. Within the Basophil/Lobularity channel the NRBC nuclei are located in the polymorphonuclear region and can be calculated by subtracting the neutrophil and eosinophil counts form the total nuclei count. Therefore for every sample 4 NRBC counts are generated,

Appendix XVII TOTAL PROTEIN (TP)

XVII. A. EXPLANATION OF THE TEST

Serum proteins serve numerous functions, both physical and chemical. They play a general role in the maintainence of normal water distribution between tissues and the vascular compartment as well as acting as buffers for acid-base balance. In certain pathological conditions the total protein and the ratio of the individual protein fractions may change independently of one another. The total protein may be raised in dehydration, myeloma and infection. Conversely the total protein may be lowered in nephrotic syndrome (due to renal protein loss), malabsorption, hepatic or renal failure, immunoglobulin deficiency or starvation.

XVII. B. METHOD

TP reagents supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD

Biuret reagent (an alkaline solution of copper ions) reacts with the peptide bonds present in proteins or short polypeptides, to form a stable chromophore with an absorbance at 545 nm. Assuming the proportion of peptide bonds present is constant with respect to the weight of protein, comparison of the increase in absorbance with a known calibrant enables the protein concentration of the sample to be determined. Two reagent additions are used, the colour reagent being in the second reagent. This enables a sample blank correction to be made before reagent 2 addition which reduces interferences (especially from lipaemia).

XVII. C. TECHNICAL DATA

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 2 µL of sample in the test, although the minimum volume required in the specimen container is 50µL (micro sample cups) or 200µL (Vacutainers and inserts).

- *LINEARITY*

Linear to approximately 120 g/L.

- *STANDARDIZATION*

The ADVIA triglyceride method measures total glycerols and is traceable to a reference method, which uses reference materials from the National Institute of Standards and Technology (NIST), via patient sample correlation. Assigned values of Bayer Chemistry Calibrator and Bayer Assayed Chemistry Controls are traceable to this standardization.

- *REFERENCE RANGE*

Serum**:** 60 - 80 g/L

No reference ranges are available for other fluids

Appendix XVIII **ALBUMIN (ALB)**

XVIII. A. EXPLANATION OF THE TEST

Albumin is the major plasma protein. It acts as a transport protein for many metabolites and drugs including bilirubin, calcium, thyroxine and anticonvulsants. It also contributes to plasma oncotic pressure and acts as a store of circulating amino acids for protein synthesis by the tissues.

Measurement of plasma albumin provides an index of liver synthetic capacity or dietary intake and as such is a useful prognostic indicator. Measurement of albumin allows interpretation of the levels of substances which bind albumin and correction to extrapolate "free" levels (particularly calcium).

Miscellaneous fluid albumin is of use in determining whether the fluid is an exudate (high level) or transudate (low level).

XVIII. B. METHOD

Albumin reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD

The Siemens Advia method for the measurement of albumin uses Bromocresol green (BCG) which preferentially binds serum albumin causing a shift in the absorption spectrum. The increase in absorbance measured at 596 nm is directly proportional to the albumin concentration.

XVIII. C. TECHNICAL DATA

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 2 µl of sample in the test, although the minimum volume required in the specimen container is 50µl (micro sample cups) or 200µl (Vacutainers and inserts). Urinary albumin should be analysed by the separate immunoturbidimetric assay.

- *LINEARITY*

Linear from 10 to 60 g/l.

- *REFERENCE RANGE*

Serum 35 - 50 g/L.

Appendix XIX **BILIRUBIN**

XIX. A. EXPLANATION OF THE TEST

Bilirubin is the major breakdown product of haem metabolism, most of this coming from the degradation of senascent red blood cells. Plasma bilirubin is taken up by the hepatic circulation and conjugated to form a soluble glucuronide which is excreted in the bile. Normally most of the plasma bilirubin is unconjugated and transported bound to albumin, while some is conjugated (by the liver as described above) and a small proportion is thought to be covalently linked to albumin (delta bilirubin).

Total bilirubin reflects either a rate of red blood cell turnover in excess of the rate of removal, decreased hepatic conjugative capacity or impaired biliary excretion. Thus, a raised total bilirubin indicates a haemolytic or hepatic disorder.

XIX. B. METHOD

Bilirubin reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD

Bilirubin reacts under acidic conditions with vanadate ions resulting in its oxidation to biliverdin. In the presence of detergent and a citrate buffer, both conjugated and unconjugated bilirubin are oxidised. The decrease in bilirubin concentration which results from this is measured at 451 nm, and the difference in absorbance over the 5 minute reaction period is related to bilirubin concentration by comparison to a previous calibration assay.

XIX. C. TECHNICAL DATA

- *SAMPLE REQUIREMENTS*

Serum, heparinised plasma or miscellaneous fluids may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 14 µl of sample in the test, although the minimum volume required in the specimen container is 50µl (micro sample cups) or 200µl (Vacutainers and inserts).

- *LINEARITY*

Linear to 598 µmol/l.

- *REFERENCE RANGE*

The reference range for serum**:** 3 - 20 µmol/L

No reference ranges are available for other fluids

Appendix XX ASPARTATE TRANSAMINASE (AST)

XX. A. EXPLANATION OF THE TEST

Aspartate transaminase (AST) is an intracellular (mitochondrial) enzyme present in most tissues, notably in the liver parenchymal cells, cardiac muscle and skeletal muscle. Raised levels of AST are associated with cell necrosis or increased cell leakage. AST is therefore markedly raised in hepatic inflammation or tissue damage and skeletal muscle damage and may be moderately raised in myocardial infarction. AST is thus a reasonably sensitive indicator of hepatitis, liver necrosis or liver transplant rejection and a relatively late indicator of myocardial infarction.

XX. B. METHOD

AST reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD.

The reaction is initiated by ~~the addition~~ of the substrate (2-oxoglutarate - contained in reagent 2) to the patient sample/co-enzyme (reagent 1) mixture.

$$\text{L-aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate} + \text{L-glutamate}$$

$$\text{oxaloacetate} + \text{NADH} \xrightarrow{\text{malate dehydrogenase}} \text{malate} + \text{NAD}^+$$

The rate of NADH consumption by the 2nd (linked) reaction is monitored at 340 nm and is directly proportional to the AST activity in the sample. Pre-incubation of the sample with the co-enzyme reagent removes any endogenous NADH by the conversion of pyruvate to lactate by lactate dehydrogenase which is added to the reagent.

XX. C. TECHNICAL DATA

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 20 µl of sample in the test, although the minimum volume required in the specimen container is 50µl (micro sample cups) or 200µl (Vacutainers and inserts).

- *LINEARITY*

The ADVIA 2400 performs a number of checks on the validity of the reaction curve to ensure linearity and to check for substrate depletion. There is also the concept of 'point forwarding'. This means that an assay which exceeds absorbance limits over the normal measuring range of the assay uses an earlier set of measuring points to obtain an acceptable reaction rate. The absorbance limits used are also variable according to the background absorbance of the sample and first reagent. This means that an exact linearity limit cannot be stated and that very few dilutions are necessary.

- *REFERENCE RANGE*

The reference range for AST is 5 - 50 IU/L

Appendix XXI ALKALINE PHOSPHATASE

XXI. A. EXPLANATION OF THE TEST

The enzyme alkaline phosphatase (ALP) is an indicator of bone formation and as such is raised in association with increased bone turnover. The production of hepatic ALP is increased in cholestasis by bile induction and thus a raised ALP may also act as a marker for cholestasis. ALP is also produced by the placenta, hence the activity is normally increased during pregnancy

XXI. B. METHOD

ALP reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD.

Para-nitrophenyl phosphate (PNPP) is an artificial substrate for ALP. ALP hydrolyses PNPP to form free phosphate and para-nitrophenol which is highly coloured (yellow). The reaction is monitored kinetically at 410 nm, the rate of increase in absorbance being directly proportional to ALP activity in the sample and expressed as IU/L by means of a conversion factor. 4-aminomethylpropanol (AMP) buffer maintains the required alkaline pH and acts as a phoshate acceptor, thus driving the reaction. Magnesium and zinc ions are present as ALP co-factors.

XXI. C. TECHNICAL DATA

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 2 µL of sample in the test, although the minimum volume required in the specimen container is 50µL (micro sample cups) or 200µL (Vacutainers and inserts).

- *LINEARITY*

The ADVIA 2400 performs a number of checks on the validity of the reaction curve to ensure linearity and to check for substrate depletion. There is also the concept of 'point forwarding'. This means that an assay which exceeds absorbance limits over the normal measuring range of the assay uses an earlier set of measuring points to

obtain an acceptable reaction rate. The absorbance limits used are also variable according to the background absorbance of the sample and first reagent. This means that an exact linearity limit cannot be stated and that very few dilutions are necessary

- *REFERENCE RANGE*

The reference range for alkaline phosphatase is age-dependant according to the following table:

Age range	Reference range (IU/L)
0 - 1 years	126 - 524
1 - 3 years	129 - 291
3 - 6 years	134 - 346
6 - 9 years	156 - 386
9 - 11 years	120 - 488
11 - 13 years	178 - 455
13 - 15 years	116 - 483
15 - 18 years	58 - 237
18 years +	30 - 130

Appendix XXII GAMMA-GLUTAMYL TRANSFERASE (GGT)

XXII. A. EXPLANATION OF THE TEST

Gamma-glutamyl transferase (GGT) is an intracellular cytosolic enzyme present mainly in hepatic tissue. Plasma activity of GGT is raised by increased cell leakage (e.g. due to inflammation), increased cell turnover and the induction of enzyme synthesis which occurs via the cytochrome P450 system. Induction may be the result of metabolism of ethanol and certain drugs (e.g. barbiturates), and thus raised GGT activities may indicate long-term excessive alcohol consumption. As the level is not affected by changes in bone status GGT is a relatively specific and sensitive marker of obstructive and infiltrative hepatic disorders and is often seen raised in parallel with alkaline phosphatase.

XXII. B. METHOD

GGT reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD.

GGT catalyses the transfer of the gamma-glutamyl residue of the artificial substrate gamma-glutamyl-3-carboxy-4-nitroanilide to the acceptor molecule glycylglycine. This liberates 4-nitroaniline which absorbs strongly at 410 nm. The increase in absorbance of 4-nitroaniline is directly proportional to the activity of GGT in the sample and expressed as IU/l by means of a conversion factor

XXII. C. TECHNICAL DATA

- *SAMPLE REQUIREMENTS*

Serum or heparinised plasma may be used. Specimens may be refrigerated overnight or stored at room temperature in seroseparator tubes. The ADVIA 2400 uses 10 µl of sample in the test, although the minimum volume required in the specimen container is 50µl (micro sample cups) or 200µl (Vacutainers and inserts).

- *LINEARITY*

The ADVIA 2400 performs a number of checks on the validity of the reaction curve to ensure linearity and to check for substrate depletion. There is also the concept

of 'point forwarding'. This means that an assay which exceeds absorbance limits over the normal measuring range of the assay uses an earlier set of measuring points to obtain an acceptable reaction rate. The absorbance limits used are also variable according to the background absorbance of the sample and first reagent. This means that an exact linearity limit cannot be stated and that very few dilutions are necessary.

- *REFERENCE RANGES*

Female: 1 - 45 IU/L

Male: 1 - 55 IU/L

Appendix XXIII **INSTRUCTIONS FOR 24H BLOOD PRESSURE MONITORING**

24 hour Blood Pressure Monitoring - Subject Information Sheet

The aim of this test is to monitor what is happening to your blood pressure over a 24 hour period. Please make your measurement 2 or 3 days before your visit to FWB. Please try to make the measurement over 25h, as the first hour when you are getting used to wearing the monitor does not always give us a representative reading. You will be asked to do your measurement at approximately the same time before each visit.

We will provide you with a diary card to fill in on the day of measurements, please fill in your activity level at the time of measurement, the time you go to bed, the time you get up and any unusual circumstances.

Procedure:
Night and day; the blood pressure cuff has two settings; a day time setting and a night time setting. The monitor will automatically change to night time settings at 10pm and day time settings at 7am.
Frequency of cuff inflation; During the day time the cuff will inflate every 30 minutes, during the night time the cuff will inflate every 1 hr.

During a measurement:
To avoid incorrect results the arm must be kept still during measurements.
If you are standing; let your arm hang loosely, whilst keeping it still.
If you are sitting; rest your arm loosely on a table or let your arm hang loosely, whilst keeping it still.
Avoid opening and closing your hand during the measurement, and do not move your fingers.
Ensure the air tube is not kinked while the measurement is being taken.
If you are driving when a measurement starts, continue to drive normally and do not worry about keeping the arm still (please make a note on the diary card if this happens), or turn the monitor off whilst you are driving.
While you are asleep, place the recorder on its side so that the air tube will not kink.
In the event of failed measurements the monitor will repeat the measurement.

Between measurements:
Engage in normal activities.
Check that the yellow mark is still in position as the cuff may move during the day.

Using the blood pressure monitor

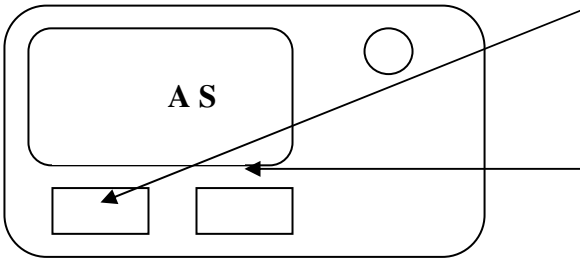
How to put the cuff on upper arm:
Put the cuff on the upper left arm so that the position of the yellow marking of the cuff is on the artery; the opposite side to your elbow.
The cuff should be wrapped on the arm tightly, but still allow a finger to slide between the cuff and the arm.

How to turn the monitor on and off:
To start the 24-hr measurement press and hold the black 'AUTO ON/OFF' button until it bleeps and the letter 'A' appears on the display.
To turn the monitor off or pause it when you take the cuff off (during bathing or exercise) press and hold down the 'AUTO ON/OFF' button until the letter 'A' is no longer on the display.

When you want to restart it press and hold the black 'AUTO ON/OFF' button until it bleeps and the letter 'A' appears on the display.
To deflate the cuff or terminate a recording press the red START/STOP button.
If the letter 'E' appears on the display at any time this means that there is an error and you will need to contact Aseel or Sarah. If the letter 'B' appears on the display at any time this means that the batteries need replacing.

If water gets into the unit, please remove the cuff and do not continue using the recorder. Turn off the unit and remove the batteries.

Some notes on operation:
Display



Red START/STOP button - can be used during a measurement to terminate a measurement and deflate

Black AUTO ON/OFF button – this will switch the monitor on to automatic mode (press for 3 seconds, until the letter 'A' appears on the display) or to cancel

A - This is displayed when the unit is in automatic measurement mode
B – This is displayed when battery capacity is low
S – This is displayed when the unit is in sleep interval measurement mode

IF YOU EXPERIENCE ANY PAIN OR AN EXTREMELY UNPLEASANT SENSATION DURING MEASUREMENTS PLEASE TURN OFF THE UNIT IMMEDIATELY, USING THE

In case of any problems please call Aseel or Sarah on 020 7848 4594 or in emergency 07789002228 or 07964919960.

Appendix XXIV **DIARY CARD FOR 24H BLOOD PRESSURE MONITORING**

Diary Card

Name: **Date:**

Please fill in your activity level at the time of measurement, the time you go to bed, the time you get up and any unusual circumstances.

Time you go to sleep…… Time you wake up…….....

Did you take any exercise Y / N If Yes at what time……....

Time	**Activity level (please circle)**			**Other**
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
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	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
Time	**Activity level**			**Other**
	Lying down	Sitting	Walking	

	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	

Notes:

Brief Instructions: Refer to the *24 hour Blood Pressure Monitoring - Subject Information Sheet* for full instructions. During the day time (7am – 10pm), the cuff will inflate every 30 minutes and during the night time (10pm-7am) every 1 hr. Between measurements engage in normal activities, also check that the yellow mark is still in position as the cuff may move during the day. The arm must be kept still during measurements. To start the 24-hr measurement press and hold the black 'AUTO ON/OFF' button until it bleeps and the letter 'A' appears on the display. To turn the monitor off or pause it whilst bathing etc press and hold down the 'AUTO ON/OFF' button until the letter 'A' is no longer on the display. To deflate the cuff or terminate a recording press the red START/STOP button.

In case of any problems please call Aseel or Sarah on 020 7848 4594.

IF YOU EXPERIENCE ANY PAIN OR AN EXTREMELY UNPLEASANT SENSATION DURING MEASUREMENTS PLEASE TURN OFF THE UNIT IMMEDIATELY, USING THE ON/OFF KEY AND REMOVE THE CUFF.

Appendix XXV **VISIT REMINDER FOR PARTICIPANT**

Dear …

We would like to remind you that your appointment for the EPA and DHA Trial is at **9 am*** tomorrow morning.

You will need to arrive at the metabolic unit on the 4th floor of Franklin-Wilkins building at the time stated above. There will be arrow signs on the 4th floor to guide you. Please use the phone at the end of corridor A to ring the unit on extension 4304 or 4594 when you arrive, and we will come and get you.

Please remember not to drink alcohol today and to arrive fasting, having not consumed any food or drink, except water, from **11pm*** tonight. You should also avoid coffee from midday, any strenuous exercise, and consume a low fat dinner: please avoid any fried food, red meat or cakes, but you can have fruit, veg and starch - potato, pasta, rice... - as much as you like. You could also get the ready to eat low fat meals from Sainsbury (be good to yourself) or Tesco (healthy living) for example. We strongly advice that you drink water before you arrive to avoid dehydration.

Please remember to bring back the blood pressure monitor and urine tanks, as well as the remaining capsules and the container. **We will give you new ones for the next 6 weeks of intervention**.**

Thanks again for taking part in the study,

We are looking forward to seeing you tomorrow,

Sarah & Aseel

* Times were adapted so the subject fasts for the 12h preceding the visit.
** Sentence was removed for the reminder of the second visit.

Appendix XXVI **KCL PARTICIPANT RECORD SHEET**

PANT RECORD SHEET

PARTICIPANT CODE			
PARTICIPANT NAME			
D.O.B.			
DATE		**TIME**	
VISIT	1 / 2 /	**INTERVENTION**	A / B / C /
PARTICIPANT WEIGHT (Kg)			
CHECK	Low fat meal last night (< 10 g fat) YES / NO Fasted since 10 pm last night YES / NO No caffeine since midday yesterday YES / NO No alcohol all day yesterday YES / NO No exercise all day yesterday and today YES / NO No medications taken since the beginning of the study YES / NO No supplements taken since the beginning of the study YES / NO Compliance to oily fish consumption restriction YES / NO Compliance to capsules intake 5/d YES / NO Advise to sip water		
Blood taken 47ml	YES / NO Order of draw: 1. 8.5 ml serum (Adpn...) 2. 8.5 ml serum (sdLDL, Nox, IF...) 3. 4 ml LH 4. 4 ml FX 5. 4.5 ml Citrate (IsoP) 6. 4.5 ml Citrate (IsoP) 7. 4.5 ml EDTA (EPC) – RELEASE TOURNIQUET 8. 4.5 ml EDTA (PGI2) 9. 4.5 ml Citrate (PMA)		
Urine sample collected	YES / NO		
24h Blood pressure and diary card	Collected: YES / NO Data transferred: YES / NO Start: … : … Finish: … : … Sleep: … : … to … : …		

Seated blood pressure (mmHg) To be taken at 2-5 minute intervals	Reading 1: (S) Pulse: (D) Reading 2: (S) Pulse: (D) Reading 3: (S) Pulse: (D) Average of reading 2 & 3: (S) Pulse: (D)

Supine position:

DVP	1	2	3	Average
HR (bpm)				
SI (m/s)				
RI (%)				
PPT (ms)				

PWA	1	2	3	Average
SBP(mmHg)				
DBP(mmHg)				
MAP				
HR(bpm)				
CSp(mmHg)				
CDp(mmHg)				
Peripheral AIx %				
Central AIx %				
P1 (mmHg)				
T1 (ms)				

Notes:

Appendix XXVII **PREPARATION OF PLASMA SAMPLES FOR F2-ISOPROSTANE ANALYSIS**

XXVII. A. SAMPLE PREPARATION

Blood was collected into 4.5 ml citrate tubes chilled on ice beforehand. Each citrated blood sample (4.5 mL) was spiked with 34 µl of 2mM indomethacin solution and mixed several times. Blood samples were then allowed to stand on ice for 30 min and centrifuged at 2400 g for 15 min at 4°C. Plasma was aspirated and transferred to cryovials (2 mL each). 6 µl of 5mM BHT ethanolic solution were then added to each 2 mL aliquots before storage at -80°C

XXVII. B. SOLUTION PREPARATION

Indomethacin is a 2 mM (0.716 mg/ml) working solution in 5% w/v aqueous $NaHCO_3$ (sodium hydrogen carbonate) solution. 5 g $NaHCO_3$ were dissolved in 10 mL distilled water (water bath). 7.16 mg indomethacin (± 0.5 mg) were added and dissolved again using the water bath.

Indomethacin solution was prepared prior to blood collection and discarded after 24h.

BHT (butylated hydroxytoluene) was prepared as a 5 mM (1.102 mg/ml) stock solution in ethanol. 11.02 mg BHT (± 0.5 mg) were dissolved in 10 ml ethanol (or 27.55 mg in 25 mL).

BHT solution was stored for 2 weeks at 4°C before being discarded.

Appendix XXVIII **BLOOD HANDLING PROTOCOL (STUDY DAYS)**

TUBES	Order Draw	ICE/ RT	CENTRIFUGE	SEPARATION		ANALYTES	Labels	ANALYSIS	STORAGE
				ul / vial	vials				
4 ml fluoride oxalate (grey)	4	ICE	15m X 1300g 4°C	3	1	Glucose	Glc	KCL– ILAB	-40°C
				3	1	Spare	Sp		-40°C
8.5 ml serum	1	RT	15m X 1300g 4°C	200	1	Adiponectin	Adpn	Unilever	-80°C
				200	1	CRP	CRP	KCH	-40°C
				200	1	Resistin	Res	Unilever	-40°C
				200	1	spare	Sp1		-40°C
8.5 ml serum	2	RT	15m X 1300g 4°C	200	1	sdLDL	sdLDL		-80°C
				200	1	TAG, NEFA, cholesterol, HDL	FLIP	KCL - ILAB	-80°C
				200	1	MMP-9	MMP9		-80°C
				500	1	NOx	NOx	KCL	-80°C
				200	2	Inflammatory Markers	IF		-80°C
				200	2	Spare	Serum Sp2,3		-80°C
				200	1	Apolipoprotein B100	ApoB100	KCL	-80°C
				200	1	Apolipoprotein A1	ApoA1		-80°C
4 ml for insulin (LH)	3	ICE	15m X 1300g 4°C	300	1	Insulin	Ins	KCH	-40°C
				300	1	Spare	Sp		-40°C
4.5 ml EDTA	8	ICE	15m X 1300g 4°C	500	1	PFA	PFA	KCL - GLC	-40°C
				600	1	prostacyclin	PGI2	KCL	-80°C
				200	2	spare	EDTA Sp1,2		-80°C
4.5 ml EDTA	7	ICE	Before centrifugation	~1000		EPC	EPC	KCL	Fresh WB
			15m X 1300g 4°C	pellet		Erythrocyte lipids + spare	Er Lip1,2		Fresh cells/ -40°C
				500	1	spare	EDTA Sp3		-80°C
3 *4.5 ml Citrate (blue)	5	ICE	15m X 1300g @ 21°C	1500	1	isoprostane	IsoPs	KCL	-80°C
	6			1500	2	Spare	CitSp1,2		-80°C
	9	RT	Before centrifugation	~500		PMA	PMA	KCL	Fresh WB
			15m X 1300g @ 21°C	500	1	spare	CitSp3		-80°C

Appendix XXIX NON ESTERIFIED FATTY ACIDS (NEFA)

XXIX. A. EXPLANATION OF THE TEST

Non-esterified fatty acid (NEFA) in the blood is bound to albumin and is used an important energy source of peripheral tissues. The amount of NEFA in serum depends on a balance between intake by the liver and peripheral tissues, and the release from adipose tissue. NEFA is decreased by physical exercise, and increased by starvation, cold, fear or smoking.

NEFAs play a key role in diabetes mellitus and insulin resistance. The occurrence of insulin resistance, glucose intolerance, high blood pressure, obesity and lipid disorders is described as metabolic syndrome. An increase in NEFA plasma concentration is reported for insulin resistance. NEFA, therefore is a risk marker of diabetes mellitus type 2 development.

XXIX. B. METHOD

NEFA Assay kit is supplied by WAKO Chemicals Gmbh, Fuggerstrabe 12, D-41468 Neuss, Germany

NEFA in the sample is converted to acyl coenzyme A, AMP and pyrophosphoric acid (PPi) by the action of acyl coenzyme A synthetase (ACS), under coexistence with coenzyme A (CoA) and ATP. Obtained acyl coenzyme A is oxidised and yields 2,3-trans-Enoyl-CoA and hydrogen peroxide by the action of acyl coenzyme A oxidase (ACOD). In the presence of peroxidase (POD), the hydrogen peroxide formed yields a blue purple pigment by quantitative oxidation condensation with 3-Methyl-N-Ethyl-N-(ß-Hydroxyethyl)-Aniline (MEHA) and 4-amino-antipyrine (4-AA).

Non-esterified fatty acids concentration is obtained by measuring absorbance of the blue purple colour.

$$\text{RCOOH + ATP + CoA} \xrightarrow{\text{ACS}} \text{Acyl-CoA + AMP + PPi}$$

$$\text{Acyl-CoA + } O_2 \xrightarrow{\text{ACOD}} \text{2,3-trans-Enoyl-CoA + } H_2O_2$$

XXIX. C. TECHNICAL DATA

- *PRECISION*

Intra-assay & inter-assay precision is not more than 1.5%

- *SAMPLE REQUIREMENTS*

Serum or EDTA plasma are recommended and they should be separated from cells within 3 hours of collection. Specimens may be stored at +2 to +8° for up to 2 days or at -20°C for up to 3 months. For longer storage -80°C is recommended.

- *SENSITIVITY*

The minimum detectable concentration of NEFA is 0.01mmol/L.

- *REFERENCE RANGE*

Male:- 0.1 – 0.60 mmol/L

Female:- 0.1 – 0.45 mmol/L

Appendix XXX **INSULIN**

XXX. A. EXPLANATION OF THE TEST

Insulin may be used, along with glucose and C-peptide levels, to help diagnose insulinomas and to help diagnose the cause of documented acute or chronic hypoglycemia. Insulin and C-peptide levels may also be used to monitor the amount of endogenous insulin produced by the beta cells, to check for insulin resistance, and to help determine when a type 2 diabetic might need to start taking insulin to supplement oral medications.

XXX. B. METHOD

Insulin reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, Gu16 8QD.

The Siemens Advia Centaur assay is a two-site sandwich immunoassay using direct chemiluminometric technology which uses constant amounts of two antibodies. Sample is incubated with two insulin-specific antibodies. The first is in the Lite reagent, is a monoclonal mouse anti-insulin antibody labelled with acridinium ester. The second antibody, in the solid phase, is a monoclonal mouse anti-insulin antibody, which is covalently coupled to paramagnetic particles. Insulin forms a sandwich between the two antibodies. After the incubation a magnetic field is applied causing the solid phase (including the sandwich) to be held at the site of the reaction cuvette while the liquid phase is aspirated. The cuvette is then washed with deionised water. Acid reagent (containing hydrogen peroxide) is then added to the cuvette to begin the light emission reaction with the acridinium ester. The cuvette is then moved to the luminometer and base reagent is added to enhance the light reaction. Light intensity is measured immediately and converted to relative light units. This has a direct proportional relationship with the insulin concentration.

XXX. C. TECHNICAL DATA

- *INTRA-ASSAY PRECISION*

	Level 1	Level 2	Level 3
Mean (mU/L)	14.68	45.72	124.51
CV (%)	4.6	3.2	3.3

- *INTER-ASSAY PRECISION*

	Level 1	Level 2	Level 3
Mean (mU/L)	14.68	45.72	124.51
CV (%)	5.9	2.6	4.8

- *SAMPLE REQUIREMENTS*

The specimen should be serum from a plain or gel separator vacutainer. The usual precautions for venipuncture should be observed.

Serum should be placed in the freezer at -20°C until assayed. Avoid repeat freeze-thaw cycles.

To assay the sample in duplicate a minimum of 50 µL is required. However, the sample tube should contain at least a volume of 250 µL to account for the dead volume and dilution.

- *SENSITIVITY*

The minimum detectable concentration is 0.5 mU/L.

- *LINEARITY*

Serum concentrations up to 300 mU/L.

- *STANDARDIZATION*

The ADVIA Centaur Insulin assay standardization is traceable to World Health Organization (WHO) 1st IRP 66/304 Assigned values for calibrators are traceable to this standardization.

Appendix XXXI **GC-NCI-MS PROCEDURE FOR ISOPROSTANE ANALYSIS**

Instrument: Agilent Technologies 6890N network Gas Chromatograph system equipped with 7683 series autoinjector, PTV (Gerstel) Inlet and 5673 inert mass selective detector with chemical ionization module

Data/Control System: Agilent Enhanced MSD Chem Station running on Windows XP Professional

XXXI. A. OPERATING PARAMETERS

- Column Type: Supelco SPB-1701 capillary.
- Column Dimensions: 30m length x 250µm (i.d) x 0.25µm film thickness
- Injection Liner: Open Baffle
- Inlet Type: Programmable Temperature Vaporization (PTV)
- Carrier Gas: Helium (Air products GC grade)
- Column Flow: 1.5ml/min (Constant flow mode)
- Injection Mode: Solvent vent
- Inlet Temperature Programme: 50C, 0.5min., then↑ 600C/min. to 300min., 2 minutes
- Pneumatics control: Vent pressure 1.0psi, vent flow 50ml/min, vent end time 0.4 min. purge time 1.5min.
- Injection Volume: 10µl total as 5x 2µl, 1s delay time
- Oven Programme: Initial Temp.80C, 1.8min. then↑ 34 C/min. to 235C then ↑ 10.3C/min. to 280C, 10min.
- Interface/Source/Quad Temperatures: 280C/150C/150C
- EM Offset: 400 above autotune
- Solvent Delay: 12min.
- Tune File: ncich4.u
- Acquisition Mode: NCI SIM
- Resolution: Low
- Dwell time per ion: 120ms
- Ion masses: 569, 573 corresponding to carboxylate anion (M-181) via a dissociative electron capture mechanism

XXXI. B. PRE-START CHECKS

Before setting up for a run ensure an adequate supply of the following :

- Helium (check cylinder guage)
- Methane Reagent Gas (check pressure gauge and replace cylinder if necessary)
- Isooctane in autoinjector wash vials (Top up if necessary)

XXXI. C. STARTUP (FROM STANDBY STATE)

- Depress Gas A button on flow control module to allow Methane to enter the system
- Depress adjacent Purge button to flush out system with Methane at maximum
- Allow system to purge for a few hours before switching off the purge (press purge button again) and allowing gas to flow at 40.
- From the PC load the method file **PGF-NCI**. This will automatically download the operating parameters to the GC-MS system.

- Perform a tune if necessary.

XXXI. D. SETTING UP AND INITIATING A RUN

- Load autoinjector vial rack with the sample batch (standards at front).
- Using the Chem Station Sequence Editor create a sequence i.e a list of samples to be injected in a defined order, together with the methods under which the data is to be acquired and the files for data storage.
- Click on run sequence command to execute the analysis schedule.

XXXI. E. SHUTDOWN (TO STANDBY)

- Following completion of run, turn off reagent gas and leave system in standby state.

XXXI. F. CHROMATOGRAPHIC DATA EVALUATION

- Check chromatograms to ensure that the peaks have been correctly assigned. If retention times have drifted outside of the preselected retention time windows then it will be necessary to edit the peak table and reprocess the data.
- If necessary adjust integration settings to improve chromatograms.

XXXI. G. CALCULATIONS

- Construct a calibration graph for each component i.e a plot of ratio of peak area of component of interest to that of internal standard against concentration of component in standard. Perform linear regression analysis to determine the best fit line for the calibration data. Using the corresponding area ratios for the samples interpolate concentration values using the equation of the regression line.

Appendix XXXII **REVIEW PAPER**

The differential effects of EPA and DHA on cardiovascular risk factors.

Cottin SC, Sanders TA, Hall WL.

Proc Nutr Soc (2011) May;**70**(2):215-31. Epub 2011 Feb 24.

Proceedings of the Nutrition Society, Page 1 of 17 doi:10.1017/S0029665111000061

The Summer Meeting of the Nutrition Society hosted by the Scottish Section was held at Heriot-Watt University, Edinburgh on 28 June–1 July 2010

Conference on 'Nutrition and health: cell to community'

Postgraduate Symposium
The differential effects of EPA and DHA on cardiovascular risk factors

S. C. Cottin*, T. A. Sanders and W. L. Hall
Diabetes and Nutritional Sciences Division, School of Medicine, King's College London, 150 Stamford Street, London SE1 9NH, UK

Compelling evidence exists for the cardioprotective benefits resulting from consumption of fatty acids from fish oils, EPA (20:5*n*-3) and DHA (22:6*n*-3). EPA and DHA alter membrane fluidity, interact with transcription factors such as PPAR and sterol regulatory element binding protein, and are substrates for enzymes including cyclooxygenase, lipoxygenase and cytochrome P450. As a result, fish oils may improve cardiovascular health by altering lipid metabolism, inducing haemodynamic changes, decreasing arrhythmias, modulating platelet function, improving endothelial function and inhibiting inflammatory pathways. The independent effects of EPA and DHA are poorly understood. While both EPA and DHA decrease TAG levels, only DHA appears to increase HDL and LDL particle size. Evidence to date suggests that DHA is more efficient in decreasing blood pressure, heart rate and platelet aggregation compared to EPA. Fish oil consumption appears to improve arterial compliance and endothelial function; it is not yet clear as to whether differences exist between EPA and DHA in their vascular effects. In contrast, the beneficial effect of fish oils on inflammation and insulin sensitivity observed *in vitro* and in animal studies has not been confirmed in human subjects. Further investigation to clarify the relative effects of consuming EPA and DHA at a range of doses would enable elaboration of current understanding regarding cardioprotective effects of consuming oily fish and algal sources of long chain *n*-3 PUFA, and provide clearer evidence for the clinical therapeutic potential of consuming either EPA or DHA-rich oils.

EPA: DHA: *n*-3 fatty acids: Cardiovascular risk: Vascular function

In the late 1970s, Dyerberg and Bang[1] were the first to highlight the cardioprotective effect of dietary long chain *n*-3 PUFA (*n*-3 LCP) present in oily fish in the Inuit population. It is now widely accepted that habitual oily fish and fish oil intake decreases the risk of CVD[2,3] such as fatal CHD[4,5] and stroke[5,6]. Over the past 30 years, the mechanisms by which fish oils improve cardiovascular health have been extensively investigated, showing anti-inflammatory, anti-arrhythmic and anti-aggregatory effects, as well as an improvement in endothelial function (EF). Responding to the abundance of evidence, national and international organisations encourage an increased fish oil consumption[7,8]. *n*-3 LCP from fish oils include EPA (20:5*n*-3) and DHA (22:6*n*-3), and have been developed commercially as dietary supplements. Recent evidence from randomised controlled trials has produced equivocal results[9–11]. Heterogeneity of the studies in terms of dosage, duration, population target, sample size, as well as the relative amount of EPA and DHA used in supplements could account for the variability of the results. Since the appearance of purified forms of DHA on the market in the 1990s, researchers have started to investigate the differential effects of EPA and DHA on cardiovascular health. However, the number of human studies is still limited in this field and the independent effects of EPA and DHA on various cardiovascular outcomes are yet to be firmly

Abbreviations: AA, arachidonic acid; α-LNA, α-linolenic acid; BP, blood pressure; COX, cyclooxygenase; CRP, C-reactive protein; CYP450, cytochrome P450; EF, endothelial function; FMD, flow-mediated dilation; HMG, 3-hydroxy-3-methylglutaryl; HR, heart rate; HRV, HR variability; *n*-3 LCP, long chain *n*-3 PUFA; LOX, lipoxygenase; LT, leukotriene; NOS, NO synthase; Rv, resolvin; SREBP, sterol regulatory element binding protein; TX, thromboxane.

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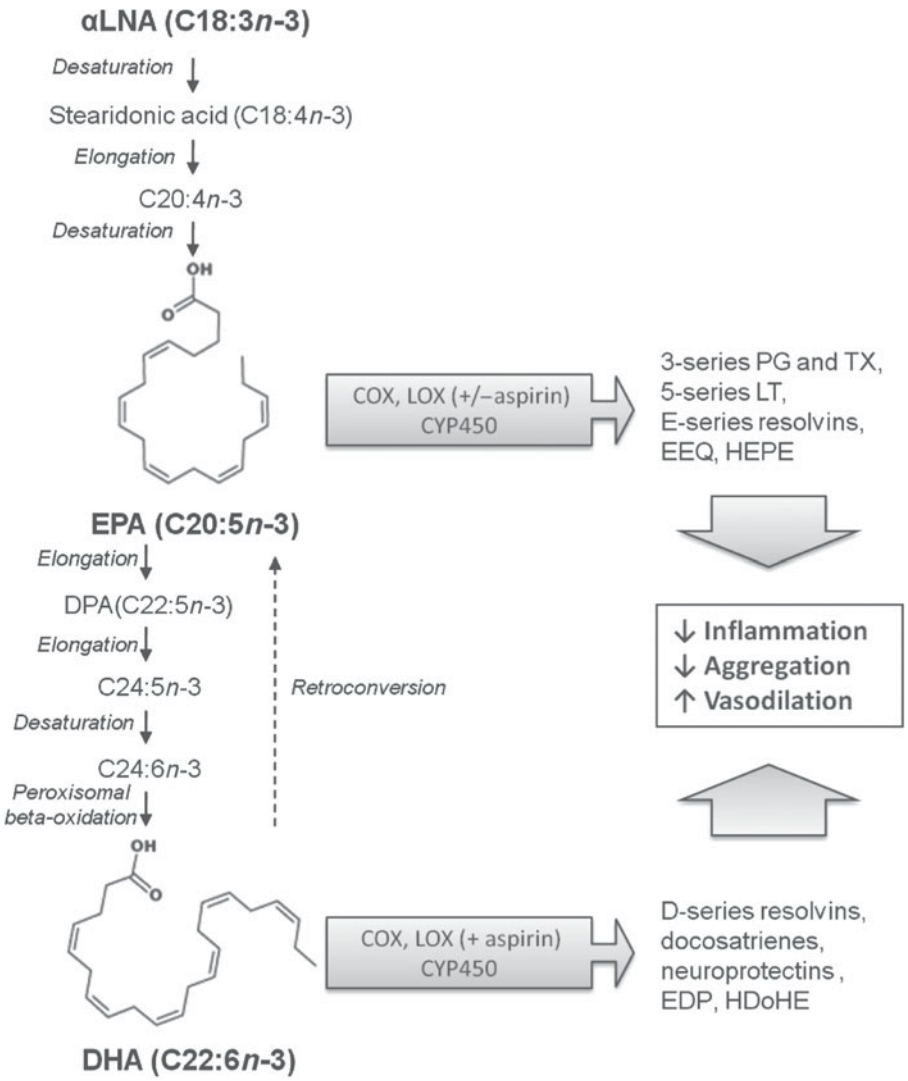

Fig. 1. Outline of the formation of EPA and DHA and their metabolites. α-LNA, α-linolenic acid; DPA, docosapentaenoic acid; COX, cyclooxygenase; LOX, lipoxygenase; CYP450, cytochrome P450 enzymes; TX, thromboxanes; LT, leukotriene; EEQ, epoxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; EDP, epoxydocosapentaenoic acid; HDoHE, hydroxydocosahexaenoic acid.

established. Further understanding in this field is needed to define optimal doses of EPA and/or DHA in order to target different metabolic disorders, and to assess the relative efficiency of algal DHA, which could be used as a source of *n*-3 LCP in vegetarians.

Structure, formation and metabolism of EPA and DHA

EPA and DHA are derived from another *n*-3 PUFA, α-linolenic acid (α-LNA; 18 : 3*n*-3) (Fig. 1), which is found in common vegetable oils, such as linseed or walnut oils. α-LNA is an essential fatty acid, i.e. it has to be provided in the diet as human subjects are unable to synthesise it. Some studies suggested that α-LNA has cardioprotective effects, but evidence is not as robust as for EPA and DHA and there are insufficient data to encourage increasing α-LNA consumption[12] in order to reduce cardiovascular risk. Human subjects can only convert α-LNA to longer-chain *n*-3 LCP at a very low rate, especially DHA[13,14], and the reduced potency or absence of effect of dietary α-LNA in improving cardiovascular risk factors suggests that dietary intake of *n*-3 fatty acids in the form of oily fish or supplements is desirable for optimal health. Cardioprotective benefits of α-LNA are mainly attributed to competition for Δ6-desaturase with linoleic acid (C18 : 2*n*-6), found in abundance in vegetable oils, seeds and nuts, and a precursor for arachidonic acid (AA; C20 : 4*n*-6), also directly obtained from animal sources including meat, eggs and dairy products, leading to production of more EPA and less AA. EPA competes with AA through the cycloxygenase (COX) and lipoxygenase (LOX) pathways, leading to a set of lipid mediators that improve vasodilation and decrease inflammation, as well as aggregation. Upon the action of aspirin, EPA and DHA can be converted by the COX and LOX pathways into similar families of resolvins, E and D series, respectively[13].

Table 1. Differential effect of EPA and DHA supplementation on plasma fasting TAG levels in human subjects.

Duration (weeks)	Design and sample size	Population	Fatty acid	Form	Dose (g/d)	Effect on TAG levels	Control	Reference
	Parallel	Normolipidaemic human subjects	EPA	Spread	2·2	↓ (15%)	None	[25]
	n 74		DHA		2·3	↓ (31%)		
4	Parallel	Normolipidaemic human subjects	EPA	EE	4·8	↓ (15%, NS)	Olive oil	[28]
	n 42		DHA		4·9	↓ (22%)		
6	Parallel	Hyperlipidaemic human subjects	EPA	EE	3·84	↓ (18%)	Olive oil	[26]
	n 56		DHA		3·68	↓ (20%)		
7	Parallel	Normolipidaemic human subjects	EPA	EE	3·8	↓ (21%)	Corn oil	[24]
	n 224		DHA		3·6	↓ (26%)		
4	Crossover	Normolipidaemic human subjects	EPA	TAG	3·3	↓ (28%)	Palmolein soyabean oil mix	[30]
	n 38		DHA		3·7	↓ (19%)		
6	Parallel	T2D human subjects with treated hypertension	EPA	EE	3·84	↓ (15%)	Olive oil	[31]
	n 50		DHA	EE	3·68	↓ (19%)		
7	Parallel	Dyslipidaemic human subjects	EPA	EE	3·04	↓ (23%)	Olive oil	[32]
	n 38		DHA	EE	2·84	↓ (32%)		
4	Parallel	Normolipidaemic human subjects	EPA	EE	3·8	=	Safflower oil	[29]
	n 33		DHA	EE	3·8	=		

EE, ethyl ester; NS, non-significant; T2D: type 2 diabetes; ↓, decrease; = , no change.

In addition, both EPA and DHA compete with AA for the cytochrome P450 (CYP450) enzymes, leading to the formation of important mediators of vasodilation[15]. These EPA- and DHA-derived eicosanoids are likely to exert varying effects within the cardiovascular system.

DHA possesses a longer carbon chain and one more double bond than EPA, which is thought to be the reason for the greater influence of DHA on membrane fluidity and cholesterol content[16], and thus on the activity of membrane protein or ion channels. EPA and DHA, as well as their broad range of derivatives, may also have a differential effect on transcription factors such as PPAR[17], NF-κB[18] or sterol regulatory element binding protein (SREBP)[19], with subsequent differences in lipid metabolism, insulin sensitivity and inflammation. This review will explore the differential effects of EPA and DHA in human subjects and relate it to possible molecular mechanisms.

Effects of EPA and DHA on plasma lipid and lipoprotein metabolism

Dyslipidaemia, specifically hypertriglyceridemia, hypercholesterolemia and/or a low HDL cholesterol level, is a major risk factor for development of atherosclerosis and CVD. The cardioprotective effects of fish oils are partially attributed to their TAG-lowering action, while their effect on cholesterol levels appears weak or inexistent.

Effect of EPA and DHA on TAG levels

Raised fasting and postprandial TAG concentrations are now widely recognised as markers of cardiovascular risk[20,21]. There is strong evidence from epidemiological and intervention studies that EPA + DHA consumption decreases TAG levels[22], thus improving cardiovascular health, and this appears to be dose-dependent[23]. When administered individually for 6 weeks or more, both EPA and DHA decrease TAG levels in normolipidaemic[24,25] and hyperlipidaemic subjects[26] from 15 to 30%. Interventions of ≤4 weeks are less consistent. One study showed that 3 weeks of supplementation with EPA or fish oil, but not DHA reduced TAG levels in healthy human subjects[27]. More recently, Buckley *et al.*[28] showed that 4 weeks of supplementation with DHA significantly reduced TAG levels in normolipidaemic human subjects by 22%, while EPA decreased TAG levels by 15% without reaching significance. In another 4-week intervention in healthy human subjects, both EPA and DHA reduced postprandial TAG without affecting fasting TAG levels[29]. However, when given for a sufficient period, EPA and DHA seem to reduce triglyceridaemia with no apparent differential effect [24–26,28–32] (Table 1).

Effect of EPA and DHA on lipoprotein profiles

Fish oils generally have no effect on total cholesterol but their influence on LDL and HDL cholesterol is variable, depending on the dose, form and population. Meta-analysis of EPA + DHA supplementation studies showed a very slight increase in LDL (*n*14 009) and HDL (*n*15 106) cholesterol levels, but these were clinically insignificant[22]. The majority of studies investigating the effect of algal DHA (that also contains docosapentaenoic acid, 22 : 5*n*-3) reported a moderate but significant increase in both HDL and LDL levels[33–37]. Few studies have reported the differential effect of purified EPA and DHA from fish oils on plasma LDL and HDL cholesterol. Relatively high doses of DHA (2–4 g/d; 6–7 weeks) increased HDL levels by 4–13% in normolipidaemics, whereas similar doses of EPA had no effect[24,25]. However, DHA but not EPA (3·7 and 3·8 g/d, respectively, 6 weeks) increased total LDL by 8% in hyperlipidaemic subjects, while no significant effect was observed on total HDL levels[26]. Our recent research observed that neither EPA nor DHA (3 g/d, 6 weeks) affected TAG, HDL

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or LDL cholesterol levels in normolipidaemic young men (SC Cottin, TAB Sanders and WL Hall, unpublished results).

Beyond cholesterol levels, LDL and HDL subfractions have emerged as candidate markers of cardiovascular risk. LDL particle size correlates negatively with TAG levels and positively with HDL levels[38]. Larger HDL (HDL-2) carry more cholesterol and are more protective than their counterpart (HDL-3)[39]. In general, dietary fish oil increases HDL-2 levels[40,41], sometimes without a significant change of HDL level[42], and also decrease small dense LDL levels[41,43]. When given individually, DHA but not EPA increased both LDL and HDL particle size in hyperlipidaemic and healthy human subjects[26,27], although EPA alone also increased HDL-2:HDL-3 in hypercholesterolaemic subjects[44]. High doses of DHA alone (3 g/d, 45 d) also increased LDL particle size in hypertriglyceridaemic men[35], whereas low doses of DHA alone (0·7 g/d) increased LDL by 7 % and LDL:apoB ratio by 3·1 % in middle-aged women and men[45], suggesting an increase in LDL size.

Hypertriglyceridaemia is a result of overproduction and/or decreased catabolism of TAG-rich lipoproteins, including VLDL and chylomicrons. There is growing evidence that EPA and DHA exert their TAG lowering effects by reducing VLDL TAG release from the liver and by increasing TAG clearance from chylomicrons and VLDL particles[46], as well as altering VLDL concentration and particle size[47]. The potential molecular mechanisms have been comprehensively reviewed by Harris *et al.*[46] and notably involves the modulation of transcription factors activity, including SREBP and PPAR. SREBP-1c controls enzymes responsible for fatty acid and TAG synthesis, while SREBP-2 modulates enzymes involved in cholesterol synthesis. In animal and *in vitro* models, both EPA and DHA were reported to down-regulate SREBP-1c activity, and this was associated with a decrease in lipogenic enzymes expression[19,48,49]. In addition, both EPA and DHA are PPAR ligands and EPA and DHA stimulate β-oxidation of fatty acids through PPARα-dependent mechanisms in rats[50], thus contributing to the decrease in TAG release by the liver. Lipoprotein lipase, located in capillary endothelium, hydrolyses circulating TAG in TAG-rich lipoprotein, generating NEFA. EPA and DHA (4 g/d, 4 weeks) were equally as effective in accelerating chylomicron TAG clearance by stimulating lipoprotein lipase activity in healthy human subjects[29]; possibly via PPARγ-dependent mechanisms[51].

3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase is a key enzyme in cholesterol synthesis and is inhibited by both EPA and DHA in hepatocytes, probably through SREBP-2 dependent mechanisms[52,53]. Although HMG-CoA reductase inhibitors (statins) are well known for their hypocholesterolemic effect, this has not been a consistent outcome of fish oils consumption. However, a common mechanism for statins and EPA/DHA may be related to increasing LDL and HDL particle size[54–56]; in fact *n*-3 LCP and statins may exert a synergistic beneficial effect on lipid levels[57], and it can be postulated that EPA and DHA modulate particle size by a mechanism analogous to that of HMG-CoA reductase inhibitors[58].

In summary, both DHA and EPA reduce fasting plasma TAG concentrations with no apparent differential effect, probably by inhibiting VLDL-TAG release and increasing TAG clearance. DHA appears to increase HDL and LDL particle size through the regulation of cholesterol synthesis and lipid transfer between lipoprotein.

Effects of EPA and DHA on haemodynamics

Blood pressure

Hypertension is a strong predictor of cardiovascular risk, and there is convincing evidence that reducing blood pressure (BP) decreases the risk of total mortality, cardiovascular mortality and stroke[59,60]. Numerous epidemiological and intervention studies have demonstrated a hypotensive role of fish oils[61]. In a meta-analysis of thirty-one placebo-controlled trials, Morris and co-workers showed that fish oils reduced BP with a dose-dependent effect (systolic BP/diastolic BP: −0·66/−0·35 mm Hg/g *n*-3 fatty acids), and so is of potential benefit to patients with hypertension, atherosclerosis or hypercholesterolaemia[62]. A more recent meta-analysis of thirty-six intervention trials confirmed the hypotensive role of fish oils on both systolic BP and diastolic BP, especially in elderly and hypertensive patients, although the clinical effect of doses lower than 0·5 g/d, equivalent to one portion of oily fish a week, could not be established[63]. Few human studies have investigated the separate effects of EPA and DHA on BP; these have generally been assessed by seated office measurements, with no significant lowering effects in hypertensive, dyslipidaemic and healthy human subjects[31,32,64]. However, low doses of DHA alone (from algal sources) were shown to decrease diastolic BP in healthy subjects[65]. Ambulatory BP, where monitors are worn and take readings at regular intervals over 24 h, considered to be an estimate of the true mean BP level[66], is more sensitive than the conventional office BP in predicting cardiovascular events[67,68]. Mori and co-workers investigated the effect of 6-weeks supplementation with EPA or DHA (4 g/d) on ambulatory BP and showed that DHA but not EPA decreased both 24 h and daytime systolic and diastolic ambulatory BP in mildly hyperlipidaemic males[69].

Heart rate

A high heart rate (HR) has been long associated with cardiovascular morbidity and mortality in epidemiological studies. It is positively correlated with hypertension and has only recently emerged as an independent cardiovascular risk factor to be targeted to reduce cardiovascular events, especially in high-risk populations[70]. A meta-analysis including thirty randomised controlled trials showed that fish oil intake reduces HR, especially in populations with a high-baseline HR and when consumed for a longer intervention period[71]. This effect appears to be mediated by DHA rather than EPA: DHA alone (2·8 g/d) decreased HR by 7 % in postmenopausal women[72], and DHA but not EPA decreased HR by 3·5 beats per minute (bpm) and 2·2 bpm, in hyperlipidaemic males[69] and healthy males[64], respectively. In contrast, Woodman and co-workers showed no

significant effect of neither EPA nor DHA on HR in healthy males for similar dosage and treatment duration[31].

HR variability (HRV) is also a strong predictor of CVD, including sudden cardiac death, arrhythmic CHD and atrial fibrillation. Fish oils have shown anti-arrhythmic properties in animal studies[73], and several clinical and epidemiological studies have reported an association between an increase (improvement) of HRV and *n*-3 LCP blood cell levels and/or fish oil consumption[74–76]. However, fish oils fail to improve HRV in several other human interventions. For example, *n*-3 LCP supplementation did not increase HRV in haemodialysis patients[77], and failed to increase HRV calculated from 10 min recordings[78] or 24 h Holter recordings[79] in healthy men. Nevertheless, the authors of the latter study noted that subjects presented with a particularly high baseline HRV, and subanalysis showed a significant improvement of HRV for subjects with lower baseline values[79]. Inconsistency in the results of intervention trials might be due to variability of design, treatment duration, sample size or duration of HRV measurement; a prospective observational study (*n* 4263) reported that fish oil consumption, recorded over a year, correlated with an improvement of HRV, especially in older people[80].

As previously mentioned, the incorporation of EPA and DHA into the cell membrane influences its organisation, fluidity and permeability, as well as the activity of transmembrane proteins, including receptors, enzymes and ion channels. Both EPA and DHA were shown to modulate K, Na and Ca channel activities in myocardial cells, regulating myocyte electrical excitability and contractility[81–83]. These effects, observed in a concentration-dependent manner, are thought to be mediated by the effect of EPA and DHA on membrane fluidity[83], although other mechanisms, such as direct binding of *n*-3 LCP to the channel could be involved[84]. Furthermore, there is growing evidence from animal studies that DHA, compared to EPA, is preferentially incorporated into the myocardial cell membrane[73]. Collectively, these findings help to explain the anti-arrythmic and HR-lowering effects observed with DHA but not EPA in human subjects[69]. In addition, incorporation of DHA into the membrane of cardiomyocytes influences the beta adrenergic system to a greater extent than EPA[85], potentially an important mechanism in the hypotensive and anti-arrhythmic effects of DHA. DHA incorporation into the membrane of endothelial cells stimulates ATP release from the endothelium, increasing vasodilation by stimulating nitric oxide (NO) release[86]. The induction of NO release, together with the decrease in noradrenaline levels, is likely to be responsible for the BP-lowering effect of DHA[86].

DHA, but not EPA, seems to have lowering effects on BP and HR, very probably mediated by the increased fluidity in the membrane cardiomyocytes, potentially improving channel activity and beta adrenergic signalling. More studies are necessary to confirm this differential effect and understand the mechanisms involved.

Effects of EPA and DHA on endothelial function and arterial compliance

Endothelial dysfunction is a key early event in the development of atherosclerosis and is characterised by an imbalance between molecules produced by the endothelium, impairing vasodilation, inflammatory status and haemostasis. In human subjects, EF can be assessed by measuring plasma markers of EF, including NO and prostacyclin metabolites (the two main vasodilators) or markers of endothelial damage and/or activation, such as soluble thrombomodulin, von Willebrand factor or E-Selectin. EF can also be assessed by non-invasive techniques such as plethysmography and flow-mediated dilation (FMD) or invasive techniques like forearm blood flow, with FMD being more commonly used. These techniques can also be used to measure endothelium-independent vascular response (using NO donors, or NO synthase (NOS) inhibitors) or vasoconstrictive response. Endothelium-derived mediators influence vascular tone and structure, thus influencing arterial stiffness and microvascular function. Non-invasive techniques have been developed to measure arterial stiffness/compliance in order to assess vascular function, which include pulse wave analysis, pulse wave velocity[87] and digital volume pulse[88].

Endothelial function

Animal studies demonstrated that EF could be modulated by feeding EPA and DHA[89–91]. An observational study reported that plasma and erythrocyte DHA levels were positively associated with FMD in young smokers and young adults at greater metabolic risk[92]. Recent findings suggest that fish oil consumption can improve EF in human subjects, particularly in those with a high risk of CVD (Table 2). Supplementation with *n*-3 LCP for periods ranging from 2 weeks up to 8 months improved endothelium-dependent vasodilation, prevented vasoconstriction or augmented exercise-induced blood flow at doses ≥0·5 g/d[93–107].

The comparative effects of EPA and DHA on EF have been seldom investigated in human subjects (Table 2). Supplementation with EPA alone (1·8 g/d; 3 months) increased endothelium-dependent forearm blood flow response in untreated hypertriglyceridaemic males[108], whereas DHA alone (1·2 g/d; 6 weeks) improved endothelium-dependent FMD in hyperlipidaemic children receiving nutritional counselling[109]. Supplementation with low doses of algal DHA did not affect salbutamol-induced changes in digital volume pulse reflection index (a measure of endothelium-dependent vasodilation), but more extensively validated techniques such as FMD are required to confirm this[65]. When the vasodilatory effects of high doses of EPA and DHA (4 g/d, 6 weeks) were compared in overweight mildly hyperlipidaemic males, DHA, but not EPA, decreased vasoconstrictive responses to noradrenaline and increased vasodilatory responses to acetylcholine[97]. However, DHA (but not EPA) also increased vasodilation in response to the co-infusion of acetylcholine and N_G-monomethyl-L-arginine citrate (an NOS inhibitor), as well as sodium nitroprusside (a NO donor), suggesting that the vasodilatory effects of DHA were mainly mediated through endothelium-independent mechanisms[97]. In healthy volunteers, fish oil concentrate, but not EPA alone, increased urinary excretion of NO metabolites (nitrates/nitrites), suggesting that EPA is unlikely to be responsible for the enhancement of

Table 2. Effect of fish oils on endothelial function in human randomised controlled trials.

Duration (weeks)	Design	Population	Dosage	Measurement	Effect	Function assessed	References
Fish oils							
6	Cross-over	T2D patients *n* 34	2 g/d	Fasting FMD	=	EF (fasting)	(106)
				Fasting RH (Doppler)	=	EF (fasting)	
				Postprandial FMD	↑	EF (postprandial)	
				Postprandial RH	↑	EF (postprandial)	
12	Parallel	Elderly *n* 46	2·5 g/d	RH (mercury strain gauge plethysmography)	↑	EF	(104)
				NOx	↑	EF	
				vWF	=	ED/EA	
				E-selectin	=	ED/EA	
				Endothelin	=	ED/EA	
12	Parallel	Offsprings of T2D *n* 50	2 g/d	FMD	↑	EF	(102)
				VCAM	=	ED/EA	
				ICAM	=	ED/EA	
				E-selectin	=	ED/EA	
12	Parallel	PAD patients *n* 32	2 g/d	FMD	↑	EF	(103)
				sTM	↓	ED/EA	
24	Parallel	Lupus erythematosus patients *n* 60	3 g/d	FMD	↑	EF	(107)
2	Parallel	Healthy subjects *n* 26	1 g/d	FMD	↑	EF	(98)
				FMD + GTN	↑	Endothelium independent vasodilation	
6	Crossover	Chronic heart failure patients (>65-year-old) *n* 20	3 g/d	FBF + Ach	↑	EF	(105)
				FBF + SNP	=	Endothelium independent vasodilation	
				FBF + AT-II	=	Vasoconstriction	
				FBF + L-NAME	=	Vasoconstriction	
6	Parallel	Healthy subjects *n* 13	5 g/d	BA diameter (post contraction)	↑	EF	(101)
				BA conductance (post contraction)	↑	EF	
				BA blood flow (post contraction)	↑	EF	
32	Parallel	Healthy subjects *n* 173	1·1–1·2 g/d	Laser Doppler + Ach	↑	EF	(95)
				Laser Doppler + SNP	=	Endothelium independent vasodilation	
16	Parallel	Hypercholesterolemic subjects *n* 30	4 g/d	FMD	↑	EF	(94)
				FMD + GTN	=	Endothelium independent vasodilation	
4	Parallel	Healthy subjects *n* 29	1·5–5·9 g/d	FBF + NAd	↓	Vasoconstriction	(93)
				+ AT-II	↓	Vasoconstriction	
6	Crossover	T2D patients	3 g/d	FBF + Ach	↑	EF	(96)

Table 2 (Continued)

Duration (weeks)	Design	Population	Dosage	Measurement	Effect	Function assessed	References
		n 23		+ GTN	=	Endothelium independent vasodilation	
EPA							
12	Parallel	Untreated hypertriglyceridaemic subjects	1·8 g/d	FBF + Ach	↑	EF	(108)
		n 15		FBF + SNP	=	Endothelium independent vasodilation	
DHA							
6	Crossover	Hyperlipidaemic children	1·2 g/d	FMD	↑	EF	(109)
		n 20		ADMA	=	ED/EA	
12	Crossover	Healthy subjects	0·7 g/d	sTM	=	ED/EA	(65)
		n 38		vWF	=	ED/EA	
				E-selectin	=	ED/EA	
EPA *v.* DHA							
6	Parallel	Overweight mildly hyperlipidaemic subjects	4 g/d	EPA FBF + NAd	=	Vasoconstriction	(97)
				FBF + Ach	=	EF	
				FBF + Ach + L-NMMA	=	Endothelium independent vasodilation	
				FBF + SNP	=	Endothelium independent vasodilation	
				DHA FBF + NAd	↓	Vasoconstriction	
		n 56		FBF + Ach	↑	EF	
				FBF + Ach + L-NMMA	↑	Endothelium independent vasodilation	
				FBF + SNP	↑	Endothelium independent vasodilation	

T2D, type 2 diabetes; PAD, peripheral arterial disease; CAD, coronary artery disease; FMD, flow-mediated dilation; RH, reactive hyperaemia; BA, brachial artery; EF, endothelial function; ED/EA, endothelial dysfunction/endothelial damage; NOx, nitrates/nitrites; vWF, von Willebrand factor; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; sTM, soluble thrombomodulin; FBF, forearm blood flow; Ach, acetylcholine; SNP, sodium nitroprusside (NO donor); L-NAME, nitro-L-arginine-methyl ester (NOS inhibitor); GTN, glyceryl trinitrate (NO donor); AT-II, angiotensin II; NAd, noradrenaline (norepinephrine); ADMA, asymmetrical dimethylarginine; L-NMMA, N_G-monomethyl-L-arginine.
↑, increase; ↓, decrease; =, unchanged.

NO production[110]. In summary, there are too few data to conclude whether EPA and DHA have differing effects on endothelium-dependent vasodilation, but early indications are that DHA might be more effective in improving EF.

Arterial compliance

Little is known about the influence of dietary *n*-3 LCP on arterial stiffness, although it has been observed that Japanese populations with higher intakes of *n*-3 LCP have reduced arterial stiffness[111], and the results of two randomised controlled trials indicate a beneficial effect[112,113]. Even less is known about the individual effects of either EPA or DHA. Three-month supplementation with low doses of DHA (0·7 g/d) in healthy subjects had no effect on indices of arterial stiffness using digital volume pulse, suggesting that either larger doses are necessary for measurable changes to occur during that time period, that fish oil is more effective in subjects at greater cardiovascular risk (our group also found no effects of 3 g/d EPA or DHA for 6 weeks in young healthy males (SC Cottin, TAB Sanders and WL Hall, unpublished results)) or lastly, that EPA rather than DHA is the active constituent of fish oil in relation to arterial stiffness[65]. In support of this, EPA supplementation (1·8 g/d; 3 months) improved pulse wave velocity and cardio-ankle vascular index in obese Japanese subjects, the latter measure being a novel index of arterial stiffness that is less influenced by BP than pulse wave velocity[114]. The same dose of EPA consumed for 12 months by hyperlipidaemic patients also prevented the increase of pulse wave velocity due to ageing, even after adjustment for gender, age and BP change[115]. However, not all evidence supports the theory that EPA is the sole active constituent of fish oil in relation to arterial stiffness; the only trial to compare the individual effects of EPA and DHA showed that 7 weeks supplementation with EPA increased systemic arterial compliance in dyslipidaemics by 36 % and DHA increased it by 27 %, with no significant differences in the size of the effect between the two groups[32].

Microvascular dysfunction, as observed in hypertensive and insulin-resistant states, is characterised by capillary rarefaction in skin and muscle[116,117]. Fish oil supplementation increased capillary density in ventricles[118] and skin (cheek pouch)[119] in hypertensive rats and hamsters, respectively, suggesting there might be beneficial effects on human microvasculature. Videomicroscopic techniques (capillaroscopy) have been developed and used in human subjects in order to look at microcirculation in the skin and oral mucosa (tongue), which are readily accessible for microscopic measurements (see comprehensive review[120]). Capillaries appear either parallel (*loops*) or perpendicular (*dot* or *comma* shaped) to the skin, and can be analysed in terms of shape (tortuousness and diameter) and number (density). Capillaroscopy also gives the possibility to assess the velocity of the erythrocytes by video or laser Doppler. However, to date, the effect of fish oil on this outcome has not been investigated. In our recent trial, neither EPA nor DHA changed finger capillary density (Capiscope; KK Technologies) in healthy young men (SC Cottin, TAB Sanders and WL Hall, unpublished results), but further research is needed in sub-populations with impaired microvascular function to ascertain whether fish oils or individual *n*-3 LCP have protective effects.

The expression of endothelial NOS is vital to EF and therefore a major factor in atherogenesis. Cell membranes are organised in microdomains, called lipid rafts, that co-localise transmembrane proteins involved in intracellular signalling pathways. When incorporated into the membrane, EPA and DHA can alter this organisation, thus modulating signalling in various types of cells[121]. In endothelial cells, both EPA and DHA were shown to alter the organisation of caveolae, a particular subset of lipid rafts, and displace endothelial NOS from caveolae, a necessary step in the activation of endothelial NOS[122,123]. This could potentially lead to an increase in NO release by the endothelium and explain the putative beneficial effects of EPA and DHA on vasodilation observed to date (Table 2). EPA and DHA probably also influence the production of the two other main vasodilators produced by the endothelium: prostacyclin and endothelium-dependent hyperpolarising factor. EPA, as a direct substrate for COX, may be converted to 3-series prostacyclin, analogue to the 2-series prostacyclin derived from AA. Both EPA and DHA increased 3-series prostacyclin production by endothelial cells to the same extent without affecting 2-series prostacyclin levels, suggesting a retroconversion from DHA to EPA[124]. In addition, EPA was shown to increase acetylcholine-induced endothelium-dependent hyperpolarising factor-mediated vasodilation in diabetic rats[125]. Endothelium-independent vasodilatory effects are mediated by the modulation of Ca^{2+} signalling in smooth muscle cells, but the mechanisms, especially with respect to the type of Ca channels involved, remain uncertain[126–129].

AA, EPA and DHA are also substrates for the CYP450 enzymes that act as monooxygenases, catalysing hydroxylation, epoxidation or allylic oxidation. CYP450-dependent derivatives include epoxyeicosatrienoic acids and hydroxyeicosatetraenoic acids, potentially important factors in the regulation of vasodilation and vasoconstriction, and modulate renal, vascular and cardiac function. CYP450 enzymes are highly regioselective and stereospecific, and several isoforms prefer *n*-3 LCP as substrates rather than *n*-6 LCP. Investigation into the physiological roles of CYP450-dependent EPA and DHA metabolites is at an early stage, but recent data suggest that CYP450-dependent mediators derived from EPA and DHA contribute to the vasodilatory and cardioprotective effects of fish oils[130]. Interestingly, different CYP450 isoforms have a different affinity, regioselectivity and stereospecificity for EPA and DHA (see comprehensive review[15]), leading to various sets of mediators that will exert varying effects on the vasculature.

In addition, EPA and DHA modulate EF through anti-inflammatory effects. When endothelial cells undergo inflammatory activation, they increase the expression of adhesion molecules, allowing the migration of leucocytes through the endothelium, an important process in the pathophysiology of atherosclerosis[131]. General patterns that emerge from *in vitro* experimental literature indicate that DHA has a greater effect than EPA in reducing endothelial inflammation. DHA tends to inhibit markers of EF, such as inflammatory cell adhesion molecules and

monocyte chemoattractant protein-1 gene and protein expression, and the adhesion of leucocytes to the endothelium, whereas EPA either up-regulated gene expression of monocyte chemoattractant protein-1 or was a weaker inhibitor of cell adhesion molecules than DHA[132–137]. The effect of DHA on vascular cell adhesion molecule-1 is likely to be mediated by the inhibition of the mobilisation of the nuclear transcription factor, NF-κB[136], which regulates the expression of numerous cytokines and other adhesion molecules.

Fish oils generally improve EF and arterial compliance in subjects at high cardiovascular risk. However, the effects of fish oils in healthy human subjects and the mechanisms (endothelium dependent and independent) by which EPA and/or DHA improve vascular function are yet to be fully established.

Effects of EPA and DHA on inflammation

Inflammation is an important process in the development of CVD; and chronic inflammation, characterised by elevated plasma levels of inflammatory markers, is commonly found in subjects at high cardiovascular risk, including type 2 diabetics and patients with CHD.

Epidemiological studies strongly suggest that fish oils have anti-inflammatory properties, and levels of *n*-3 LCP in plasma, as well as in erythrocyte membrane, negatively correlate with plasma pro-inflammatory markers, including C-reactive protein (CRP) and IL-6[138,139]. Another hypothesis for the cardioprotective effects of fish oil supplementation is the inhibition of cytokine production, as measured directly in plasma or *ex vivo*, and studies have been published that support and challenge the hypothesis that *n*-3 LCP inhibit cytokine and CRP production[102,103,140–150]. In their recent meta-analysis including twenty-one trials, Balk *et al.*[151] concluded that the effect of *n*-3 LCP, including EPA and DHA, on CRP levels in human subjects was unconvincing. In human subjects, only one study investigated the differential role of EPA and DHA (4 g/d; 6 weeks), reporting that plasma CRP, IL-6 and TNF-α remained unchanged in hypertensive type 2 diabetics[152]. When investigated separately, DHA (3 g/d, 3 months) reduced CRP at 6 weeks and IL-6 at 12 weeks of intervention in hypertriglyceridaemic subjects[153], while EPA (1·8 g/d, 8 weeks) decreased CRP levels in obese subjects[114]. Lower doses of DHA, representative of levels of intake obtained from dietary sources, failed to affect plasma CRP levels in healthy subjects[65]. Complete understanding of this topic requires intervention studies on the anti-inflammatory effects of long-term combined EPA and DHA intakes at low doses (<1·5 g/d), relevant to dietary guidelines for optimal health, and also shorter-term higher doses EPA and DHA (1·5–5 g/d), potentially important in developing therapies for at-risk patients.

Related to the observations for cytokines, an increasing dietary intake of *n*-3 LCP also modifies the eicosanoid profile in blood, reducing production of AA-derived mediators by inflammatory cells, such as leukotriene (LT) B_4 and PGE_2 and increasing EPA-derived mediators such as LTB_5 and PGE_3 (see review[154] for further information). As indicated earlier, EPA and DHA supplementation lowers the cell membrane *n*-6:*n*-3 ratio. This reduces AA availability for the production of lipid mediators through the COX and LOX pathways, including 4-series LT, 2-series PG and thromboxane (TX), while increasing the production of 5-series LT, and 3-series PG and TX[154]. 3-series EPA-derived eicosanoids, are thought to be less potent than AA-derived eicosanoids, thus contributing to the anti-inflammatory, but also the anti-aggregatory and vasodilatory effects of fish oils[154] previously described. As mentioned earlier, EPA, unlike DHA, is a direct substrate for COX and LOX for the synthesis of LT, PG and TX, which might explain why it reduces LTB_4 and PGD_2 production in macrophages to a greater extent than DHA[155]. The slight but significant effect of DHA might be due to its partial reconversion to EPA[156].

In addition, both EPA and DHA undergo a series of reactions involving COX-2 in the presence of aspirin and 5-LOX[2], leading to a novel class of lipid mediators, known as E-series resolvins (Rv) from EPA and D-series Rv and neuroprotectin D1 from DHA, which are involved in the resolution of inflammation. Although EPA- and DHA-derived compounds possess strong similarities, they exert different actions that could account for the differential effect of EPA and DHA on various processes in cardiovascular health and disease. For example, both RvE1 and RvD1 reduced the expression of vascular cell adhesion molecule-1, IL-8, macrophage inflammatory protein-1β and TNF-α by endothelial cells and reduced leucocyte transmigration through the endothelium[157]. However, the DHA-derived compound RvD1, but not the EPA-derived RvE1, decreased PGE_2 production in endothelial cells[157].

In summary, fish oils decrease inflammation, although efficacy in human studies depends on dose, population and inflammation marker chosen. Individually, DHA, and to a lesser extent EPA, have anti-inflammatory properties *in vitro* but there is insufficient information to determine whether one is more potent than the other.

Effects of EPA and DHA on thrombosis and haemostasis

While noting the cardioprotective effects of *n*-3 LCP from fish oils, Bang and Dyerberg reported that very high oily fish consumption was associated with lengthened bleeding time[1]. The anti-thrombotic action of fish oils in both healthy human subjects and people at high cardiovascular risk have been extensively investigated during the ensuing decades. Several intervention studies later confirmed the effect on bleeding time in healthy, hyperlipidaemic and patients with heart disease at generally relatively high doses of fish oils[158,159], while lower doses (<2 g/d) seem to have no significant effect[160,161]. A recent meta-analysis including twenty-four trials in type 2 diabetics (1533 subjects) concluded that fish oils reduced platelet aggregation to ADP and to collagen by 22 and 21 %, respectively[162]. In general, fish oils seem to reduce platelet aggregation and TX A2 production in response to ADP and collagen in healthy people[163,164] and in subjects with mildly raised BP and cholesterol levels[165]. However, platelet

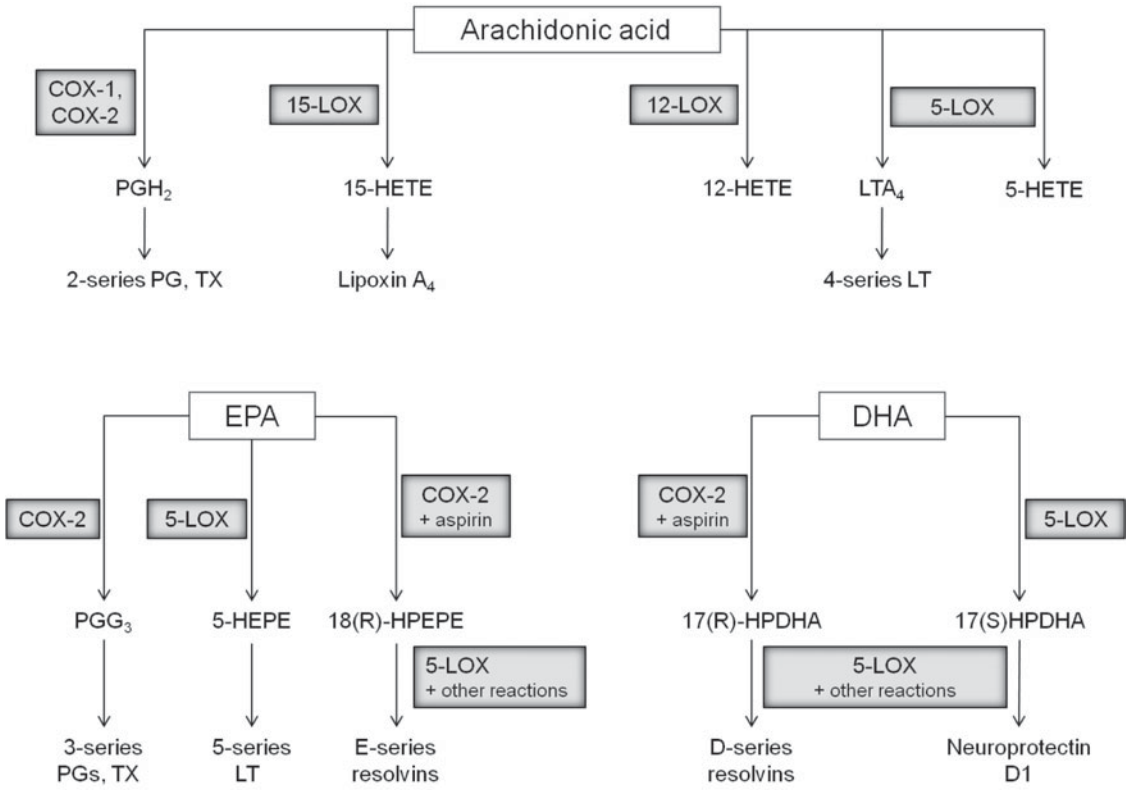

Fig. 2. Outline of the pathways of eicosanoid and lipid mediators synthesis from arachidonic acid (AA), EPA and DHA. Through cyclooxygenases (COX) and lipoxygenases (LOX), AA is converted into a set of lipid mediators including 2-series PG and thromboxanes (TX), 4-series leukotrienes (LT) and lipoxins. Competing with AA for COX and LOX enzymes, EPA is converted to 5-series LT, 3-series PG and TX, which are overall less inflammatory than the AA-derived eicosanoids. In the presence of aspirin, both EPA and DHA are substrates for COX-2, eventually leading to the formation of E- and D-series resolvins, respectively, involved in the resolution of inflammation. In addition, DHA may undergo lipoxygenation (through 5-LOX) and other reactions, producing the anti-inflammatory mediator neuroprotectin D1. HETE, hydroxyeicosatetraenoic; HPEPE, hydroperoxy-EPA; HPDHA, hydroperoxy-DHA.

aggregation is usually measured in the laboratory in response to various stimuli and there is uncertainty regarding the correlation between platelet activity *ex vivo* and *in vivo*. The effect of fish oils on *in vivo* platelet aggregation in healthy young males was recently investigated by Din and co-workers measuring platelet monocyte aggregates by flow cytometry; low doses of fish oils (1 g/d; 4 weeks) reduced platelet monocyte aggregate, while markers of platelet activation (soluble P-selectin, soluble CD40L) remained unchanged[166].

Anti-thrombotic properties of fish oils were initially attributed to EPA due to its competition with AA in the COX and LOX pathways. Accordingly, 1·8 g EPA given daily to hyperlipidaemic diabetics for 4 weeks was shown to reduce platelet- and monocyte-derived particles, as well as the expression of CD62P, CD63 and PAC-1, all markers of platelet activation[167]. Interestingly, EPA but not DHA was also able to reduce mean platelet volume, a simple marker of platelet activation in healthy subjects[168]. Four-week supplementation with 3·6 g EPA alone, daily, was also shown to decrease platelet aggregation and TX production in response to collagen in healthy males[169]. However, in agreement with animal studies[170–172], human studies suggest that DHA is a more potent anti-aggregatory agent than EPA at high doses[173,174]. More recently, 8-week supplementation with DHA alone were shown to reduce platelet aggregation to collagen in healthy males for doses as low as 0·4 g/d[175].

Due to great variability in terms of design, dose of fish oils and population type, there is inconsistency regarding the effect of fish oils on haemostatic factors in human subjects[151,176]. However, studies generally show no significant effect of *n*-3 LCP on haemostatic factors levels or activities in healthy subjects[177–179], with similar findings for algal DHA[180]. In type 2 diabetics, fish oil supplementation decreased fibrinogen levels by 10 %[162], and increased factor VII by 25 %[181], based on meta-analysis of three trials (159 participants) and two trials (116 participants), respectively. More studies are needed to clarify the independent effects of EPA and DHA on haemostatic factors.

Dietary EPA and DHA are readily incorporated into platelet membrane, leading to the formation of eicosanoids from the 3-series, less pro-thrombotic than the 2-series eicosanoids derived from AA (Fig. 2). This, in addition to the effect on platelet membrane fluidity, is likely to influence haemostatic and thrombotic processes. Competition of EPA with AA in the COX pathway (Fig. 2) reduces TXA_2 production, leading to the formation of TXA_3, a less

potent vasoconstrictor and pro-aggregatory mediator. Accordingly, both EPA and DHA decrease AA-induced TXA_2 production by platelets, while only EPA increases TXA_3 production, showing that EPA, but not DHA is a direct substrate for the COX/TX synthase complex[182]. Anti-thrombotic effects of EPA and DHA might also be endothelium dependent. 3-series prostacyclin is synthesised from EPA by endothelial cells, which adds on to the anti-aggregatory effect of 2-series prostacyclin[124]. In addition, both EPA and DHA inhibit platelet-activating factor synthesis[183] and stimulate endothelial NOS activity[122,123] in endothelial cells. The decrease in platelet-activating factor levels, as well as the increase of NO, which has anti-aggregatory properties, may also contribute to the anti-thrombotic effects of fish oils.

Fish oils seem to exert their anti-thrombotic action in human subjects by influencing platelet activation and aggregation rather than haemostatic factors levels and/or activity. DHA is more potent than EPA in reducing platelet aggregation in animals, and possibly in human subjects, possibly as a result of its greater effect on membrane fluidity[170]. In contrast to DHA, EPA is a direct substrate of COX for the synthesis of anti-inflammatory and anti-aggregatory mediators, a key factor in the inhibition of platelet activation. Further investigation is needed to specify the individual role of EPA and DHA in platelet function in human subjects, especially *in vivo*.

Insulin sensitivity and glycaemic control

Insulin resistance is characteristic of type 2 diabetes and is associated with several disorders involved in the development of CVD, including chronic inflammation, dyslipidaemia, hypertension and endothelial dysfunction.

Plasma and erythrocyte *n*-3 LCP, *n*-3:*n*-6, and especially EPA:AA ratios correlate positively with insulin sensitivity in healthy subjects and type 2 diabetics[184–186]. There is also growing evidence from animal studies that fish oil intake increases insulin sensitivity and adiponectin levels in insulin resistant rats and mice[187,188]. In contrast, intervention studies generally show little or no effect of fish oils on insulin sensitivity and glycaemic control in human subjects. Balk's meta-analysis considered healthy subjects, type 2 diabetics, hypertensives, dyslipidaemics or patients with CVD and concluded that fish oils induced no change in glycated Hb (HbA1c, eighteen trials, 578 participants) and a slight but non-significant increase in fasting blood sugar (seventeen trials, 1427 participants)[151]. This was more recently confirmed in a meta-analysis including 1075 type 2 diabetics, where the authors showed no effect of EPA and DHA on HbA1c, fasting glucose, fasting insulin or body weight[189].

There is growing evidence from animal and *in vitro* studies that both EPA and DHA, taken individually, exert an insulin-sensitising action[187,190–193]. However, the relative effect of EPA and DHA on insulin sensitivity in human subjects has been poorly investigated. EPA alone decreased insulin reactivity and increased adiponectin levels in obese Japanese, without affecting leptin levels[114]. In type 2 diabetics, EPA had no effect on adiponectin levels but an additive positive effect when combined with statin treatment[194]. To date, only three studies investigated the independent effects of EPA and DHA on insulin sensitivity in human subjects. In hyperlipidaemic subjects, both EPA and DHA (6 weeks, 4 g/d) decreased fasting insulin levels, and fasting glucose tended to increase in the EPA group, remaining unchanged following DHA supplementation[26]. In treated hypertensive type 2 diabetics, neither EPA nor DHA influenced insulin levels, secretion or sensitivity, but both increased fasting glucose[31]. More recently, Egert and co-workers confirmed that neither EPA nor DHA had an effect on HbA1c, insulin level or sensitivity in healthy subjects, although EPA showed a minor increase in glucose levels while DHA had no effect[195].

Conclusion

Numerous studies have proven the cardioprotective effects of fish oils in human subjects, showing their hypotriglyceridaemic, hypotensive, anti-arrhythmic and anti-thrombotic properties. Recent data suggest that fish oils also improve arterial stiffness and EF, and increase HDL and LDL particle size. Most studies have investigated the effect of oily fish or fish oil supplements containing mixtures of EPA and DHA, and current UK dietary guidelines recommend the consumption of one portion of oily fish a week to maintain general good health. However, over the past 20 years, there has been growing evidence that EPA and DHA exert a heterogeneous effect on various cardiovascular outcomes, which is of considerable relevance for primary and secondary cardiovascular prevention. While both EPA and DHA are able to reduce TAG levels, DHA appears responsible for the BP and HR-lowering effect of fish oils. DHA also seems to be beneficial for EF and platelet function, although an active role for EPA has not been ruled out. Although fish oils show anti-inflammatory and insulin-sensitising properties *in vitro* and in animal studies, human studies are often conflicting and efficacy remains uncertain; accordingly, neither EPA nor DHA alone showed an effect on inflammation or insulin sensitivity in human subjects, despite indications for potency *in vitro*.

The apparent efficacy of DHA in improving a number of cardiovascular risk factors, and the remaining uncertainty surrounding the actions of EPA, suggest that there is a need for *n*-3 LCP oils that are a purified or enriched source of either EPA or DHA. An increasing number of studies are being published on the cardioprotective effects of DHA TAG from algal sources, either *Crypthecodinium cohnii* or *Schizochytrium* sp. (Martek Biosciences Corporation, Columbia, MD, USA). Supplements, infant formula, infant foods and certain other food categories (dairy, bakery, eggs and non-alcoholic beverages) fortified with algal DHA are now available to buy in many countries. The potential benefits of algal DHA supplements for subgroups that have low intakes, such as vegetarians, should be a high priority for investigation. EPA TAG-enriched oils and purified EPA ethyl ester oils are available but currently a large amount of effort is being directed by industry towards the development of non-fish oil-derived EPA. As more

DHA- and EPA-only products become available, partly as a result of concern over the sustainability of fish oil supplies and partly in response to consumer demand for non-fish sources, future research can be focused on establishing the most effective doses of DHA and EPA for improvement of cardiovascular risk factors. This will inform dietary advice on the optimal intake for life-long health, and should enable a decision to be made on the most effective supplement dose to be taken over short periods to reduce risk factors such as hypertriglyceridaemia or hypertension in various at-risk populations. It will be important to bear in mind that not all individuals will respond to DHA and/or EPA in the same way, and ongoing nutrigenetic and gender research will be crucial in defining future advice regarding dietary and supplementary EPA and DHA. The role of dietary *n*-3 LCP in cardiovascular health is an area of nutritional science/medicine that has undergone more investigation than most during the past 30 or more years, yet the gaps in our understanding of this field remain substantial.

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Appendix XXXIII **CALCULATION OF FA YIELD OBTAINED AFTER BSA-FA COMPLEXATION**

Sodium salts	**C16:0 PA**	**C18:0 SA**	**C18:1(n-9) OA**	**C18:2(n-6) LA**	**C20:4(n-6) AA**	**C20:5(n-3) EPA**	**C22:6(n-3) DHA**
MW FA salt (g/mol)	278.41	306.50	304.45	302.44	326.00	324.40	350.50
MW Na (g/mol)	22.99	22.99	22.99	22.99	22.99	22.99	22.99
MW FA (g/mol)	255.42	283.51	281.46	279.45	303.01	301.41	327.51
Expected [FA] g/L	1.53	1.70	1.69	1.68	1.82	1.81	1.97
Expected [FA] mM	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Area peak Standard 1	305.00	535.39	423.00	560.96	549.16	416.00	418.00
Area peak FA 1	494.00	739.94	520.00	638.28	746.18	502.00	556.00
Observed [FA] g/L 1	1.62	1.38	1.23	1.14	1.36	1.21	1.33
Observed [FA] mM 1	6.34	4.87	4.37	4.07	4.48	4.00	4.06
Yield (%) 1	105.69	81.25	72.79	67.86	74.74	66.73	67.69
Area peak FA 2	457.00	774.45	500.00	683.31	798.50	481.00	522.00
Observed [FA] g/L 2	1.09	1.39	1.16	1.16	1.41	1.10	1.24
Area peak Standard 2	421.00	558.16	432.00	589.70	566.29	436.00	421.00
Observed [FA] mM 2	4.25	4.89	4.11	4.15	4.65	3.66	3.79
Yield (%) 2	70.83	81.57	68.54	69.11	77.56	61.00	63.10
Average yield (%)	**88.26**	**81.41**	**70.66**	**68.49**	**76.15**	**63.86**	**65.39**

MW, molecular weight; FA, fatty acids;, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid; EPA, eicospentadecanoic acid; DHA, docosahexaenoic acid

Appendix XXXIV **WESTERN BLOTTING FOR eNOS AND COX-2**

XXXIV. A. SDS-PAGE

- *GEL PREPARATION*

LOWER GEL

Reagent	10% (x1gel)	10% (x2gels)	10% (x3gels)	Final con.
4xTris-SDS pH 8.8 (mL)	2.4	4.8	7.2	1x (375mM Tris0.1% SDS)
30% Acrylamide (mL)	3.2	6.4	9.6	13, 12, 10 or 8%, respectively
dd H_2O (mL)	4	8	12	
10% APS *(uL)	35	70	105	0.04%
TEMED *(uL)	7.5	15	22.5	0.08%

* Toxic- use in hood

If necessary clean glass plates with water and wipe dry.
Wipe each plate with methanol (to precipate any protein residues)
Place smaller plate on top of larger plate and place in plate holder (with green clips) with the small plate at the front and ensure that the bottom is flat by placing the whole assembly on bare bench. Fasten clips.
Put plate holder in casting stand and fill plate cavities with MilliQ water to check if any leakage.
Whilst water is still inside the glass plates, make up lower and upper gel, minus APS and TEMED (see tables above). Invert gently to mix.
Check plates for water leakage. Pour off water, tip casting stand slightly to one side and insert filter paper between plates to remove excess water.
Insert comb into plate cavity and mark plate 1cm from bottom of comb. Remove comb.
Pipette APS and then TEMED into lower gel mixture and invert to mix.
Pipette gel mixture into each plate cavity with a plastic Pasteur until it reaches approx 2 mm above marked line (gel will shrink). Take up some of the remaining gel into the pipette (to subsequently check that the gel has set).
Slowly add ~0.5-0.6 mL water-saturated butanol into each plate cavity (to exclude air bubbles and create and sharp top edge).
Leave for 30-45 minutes until set (use gel in pipette to check this).
Make 4 mL wash solution: 1:4 dilution of Tris-SDS buffer (diluted with water).
Once the gel has set, pour off butanol (into sink).
Tip casting stand slightly to one side and carefully insert filter paper to remove excess butanol.
Place casting stand flat and pipette in washing solution (2mL each) (diluted Tris-SDS buffer).
Remove excess washing solution as above.

UPPER GEL

Reagent	Amount	Final Conc.
4xTris-SDS pH 6.8 (mL)	2	1x (375mM Tris0.1% SDS)
30% Acrylamide (mL)	1.04	4%
dd H_2O (mL)	4.88	
10% APS *(uL)	40 (70!)	0.05%
TEMED *(uL)	5 (15!)	0.06%

* Toxic- use in hood

Make upper gel in 30 mL universal according to the volumes specified in BioRad Table: Tris-SDS, water and acrylamide (order of addition not important).
Add APS and TEMED according to Table above (higher volumes set more quickly)
Using a plastic Pasteur pipette fill plate cavities to top with upper gel mixture.
Carefully remove air bubbles with the pipette.
Insert the comb at an angle and push down. The gel solution will spill over down the side of the plate. Take up some of the remaining gel into the pipette (to subsequently check that the gel has set).

- *SDS-PAGE*

Make up a 20% stock solution of 2-mercaptoethanol in 0.02% bromophenol blue (80 µL BPB + 20 µL 2ME into a 1.5 mL Eppendorf tube – remainder can be frozen and defrosted once).
Pipette 30µl of protein samples into new Eppendorfs (max volume that can be loaded)
Add 3µL 2ME/BPB.
Heat sample tubes for 3 min at 95^{o}C in heat block (add tubes individually, place metal block on top and remove individually in same order as inserted)
Make up the tank buffer: dilute 10x Tris/glycine/SDS 1: with water – add buffer to the water not the other way round (to avoid excessive bubbling).
Once the upper gel is set, remove plates from casting stand by releasing the top clip and place in the electrode assembly clamp with the combs facing inward. Push plates down into assembly clamp and clamp shut. Pour tank buffer into the middle of the assembly to check leaking.
Use a gentle rocking motion to dislodge the combs and then pull each one straight up to remove.
Attach a yellow tip to the end of a plastic Pasteur pipette and gently wash each well out by pipetting up and down twice to remove unpolymerised gel.
Use gel loading tip to load 10 µL protein ladder into lane 2 ('Rainbow marker recombinant. Load samples into wells – use wells 3-14). If a positive control is used, and there are no spare lanes, load the control in lane 1 or 15.
Fill the tank with buffer and use a plastic Pasteur pipette to remove excess bubbles
Plug leads into power pack (red to red and black to black)
Set voltage to 150 V and check there is current. Leave for approx 1 hour and 10 min. If short of time, voltage can be set to 180 V for 50 minutes.
After the running time has elapsed check that the BPB has reached the bottom of the gel.
Switch off the power pack and disconnect the leads. Pour off the buffer and take assembly back to the bench.

XXXIV. B. WESTERN BLOT

- *TRANSFER*

Cut four 5x8 cm pieces of filter paper and two 5x8 cm pieces of PVDF membrane.
Soak the membranes in absolute methanol. After that pour back the methanol.
Carefully remove each plate taking note of which is which (hold onto plates in case they fall).
Remove the outer plates and cut out the upper gel then score along the sides of the gel to free it from the inner plate (keep gel wet to prevent tearing). Cut lower corner on marker side of each gel. Place gels in transfer buffer to equilibrate for a few minutes.
Place a piece of filter paper onto the plate electrode of the transfer kit, pour on some transfer buffer.
Cut one corner of the membrane and place the membrane on the filter paper.
Scoop up a gel and place on the membrane so the corners match.
Pour on more transfer buffer and cover the membrane (membrane 1) with a second piece of filter paper.
Repeat steps 5-8 for remaining pieces of filter paper and membrane 2.
Using a roller, roll out any air bubbles working outwards from the middle of each stack and taking care not to dislodge the gel.
Pour on more transfer buffer and clip cover of transfer kit into place. Add a weight on the kit.
Connect the leads (red to red and black to black) and set the voltage on the power pack to 20 V. Check that there is current.
Whilst the transfer is taking place make up 5% skimmed milk powder in PBS/0.1% Tween (1 g milk powder in 20 mL PBS/Tween).
Following transfer (2h for eNOS), remove the membranes from the transfer kit and place each in a plastic box containing the skimmed milk/PBS/Tween solution. Label the boxes 1 and 2. (membranes may be briefly rinsed in PBS/0.1% Tween prior to refrigerating).
Block on a shaker at 1-2 rpm for 1 hour.
Place gels in a container of stain (membrane 2 on top of membrane 1) and place on shaker at ~1 rps overnight.

- *PROTEIN DETECTION*

GELS

Pour the stain solution from gel back into original bottle and add destain to the gel.
Place on a shaker at 1-2 rpm and periodically change the destain solution (3-4 times, timing not crucial) until protein bands are detectable on the gel.
Check to see if the lanes appear to have run straight or if there are any anomalies.
The gel may be preserved by wetting it with PBS/0.1% Tween and sealing in a plastic bag.

MEMBRANES

Pour off the milk solution and rapidly rinse the membranes twice in PBS/0.1% Tween.
Pour on enough TBS/0.1% Tween to cover each membrane and then place the containers on a shaker for 15 min at ~1 rpm to wash, then wash 3 x 5 min.
Meanwhile, make up 20 mL primary antibody:
Anti eNOS (Rabbit): diluted 1:1000 with PBS/0.1% Tween containing 3% BSA and a few granules of sodium azide to inhibit bacterial growth. This working solution may be refrigerated and reused approximately 10 times.
Anti α-tubulin (Rat): diluted 1:5000 with PBS/0.1% Tween containing 3% BSA and a few granules of sodium azide to inhibit bacterial growth. This working solution may be refrigerated and reused approximately 10 times.

Divide the antibody solution equally between two small plastic boxes that are just over 5x8 cm in size.
Remove membrane 1 from its box with flat forceps and carefully remove excess liquid by gently dragging the membrane over the rim of the box. Blot off remaining excess from the bottom edge of the membrane onto a blue paper towel.
Transfer membranes 1 and 2 to a small plastic boxes marked '1' and '2', respectively, containing 10 mL primary antibody.
Place boxes on a shaker at ~1 rpm for 1 hour at RT (or overnight at 4°C).
Remove each membrane from its box with flat forceps and carefully remove excess antibody by gently dragging the membrane over the rim of the box. The antibody solution should be poured back into its container and stored at 4°C. The boxes should be washed with MilliQ water and dried.
Place membranes in larger boxes and cover with TBS/0.1% Tween, rinse rapidly three times in TBS/0.1% Tween and wash for 1 x 15 min and then 3 x 5 min replacing the TBS/0.1% Tween prior to each wash.
Make up 20 mL secondary antibody:
Anti Rabbit (eNOS): 10ul diluted 1:2000 with PBS/0.1% Tween containing 3% skimmed milk powder (0.6g). This working solution is discarded after use.
Anti Rat (α-tubulin): 4ul (1:5000) in PBS/0.1%Tween 3% skimmed milk (0.6g) is used for step 12.
Divide the antibody solution equally between the two small boxes.
Place boxes on a shaker at ~1 rpm for 1 hour.
Pour off the secondary antibody and wash membranes as in step 9.
Lay a piece of blue towel on the bench. Cut two pieces of Saran wrap the first approx 20x15 cm and the second approx 40x15cm and place onto the blue towel.
Pipette 1 mL ECL detection reagent one and the same volume of reagent 2 into a 7 mL bijoux. Invert to mix.
Turn on computer and machine (open SynGene). Select Chemi Sample.
Remove each membrane from their boxes using flat forceps and dab off excess fluid onto a blue towel. Place membrane 1 onto the smaller piece of Saran wrap and membrane 2 below it (cut corner to the bottom right).
Pipette 1mL ECL reagent mix onto membrane one, start timer and 30 sec later pipette 1mL reagent onto membrane 2.
When 1 min has elapsed, pick up membrane one, dab off excess reagent and place it onto upper half of the second piece of Saran wrap. After a further 30 seconds, repeat for membrane 2 and place below membrane 1.
Fold the Saran wrap over the membranes and carefully smooth out any air bubbles. Trim off excess Saran wrap leaving a ~2 cm border around the membranes and put in the machine.
Reduce focus (eye) to ~43 and increase magnification so that membranes fill in the screen (~29). Take one picture with the door open (press the green button, then red)
Close the door and select parameters for reading (camera+files icon):
- eNOS: Number of images = 6; Exposure time = 5 min → 30min in total
- A-tubulin: Number of images = 5; Exposure time = 30 s → 2.5 min in total
- COX2:
Immediately after reading, put the membranes back in PBS-Tween for storage or too proceed to new incubation (repeat steps 2-22)
Once the membranes have been probed for α-tubulin, they may be wetted with PBS/0.1% Tween and wrapped in Saran wrap for long-term storage at 4°C.